Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda

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Declaration

I, Alice Chijioke Eziefula, declare that this thesis is my own work, and that I have acknowledged all results and quotations from the published or unpublished work of other people.

SignatureDate:  20th September 2019

Abstract

Background: After standard effective antimalarial treatment with artemisinin-based combination therapy (ACT), a proportion of individuals remain infectious to mosquitoes, enabling onward transmission. This is due to persisting gametocytes, the sexual stage of the malaria parasite. Primaquine, an 8-aminoquinoline drug, sterilizes and clears gametocytes. The World Health Organization recommends that, in areas where malaria elimination is targeted, a single dose of primaquine should be given in addition to standard antimalarial treatment. Despite its recommendation in WHO guidelines since the 1970s, the optimal dose of primaquine for this purpose had not been determined. Primaquine is associated with haemolytic toxicity in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency, a condition that is prevalent in malaria-endemic regions. This thesis presents the first dose ranging trial to assess the safety and efficacy of reducing doses of primaquine in combination with ACT to treat children with uncomplicated falciparum malaria infection.

Methods: A literature review was conducted to inform a novel, evidence-based trial design. Based in Jinja, Uganda, this randomised, double-blind, and placebo-controlled trial had four parallel treatment groups of reducing doses of primaquine plus ACT.

Results: For trial participants, a single dose of 0.4mg/kg primaquine base had non-inferior efficacy (measured by gametocyte clearance) to the WHO-recommended dose of 0.75mg/kg, whereas a dose of 0.1mg/kg was not non-inferior. There was no significant haemolysis in any of the treatment arms and the fall in haemoglobin was not associated with the dose of primaquine. However, a sub-analysis showed a dose-dependent reduction in haemoglobin in participants who were G6PD deficient (heterozygous or hemi-/homozygous genotype). Subsequently, the WHO reduced the recommended dose to 0.25mg/kg primaquine base.

Additional trial analyses that were not included in the published manuscript are presented in the thesis, together with descriptions of the trial's impact and data sharing.

Conclusion: The findings of this trial have contributed to changes in malaria elimination policy and to the prioritisation of primaquine evaluation in research agendas. This thesis puts the trial in the context of the body of evidence that has amassed since the trial results were published and highlights priorities for further research.

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I am keenly aware of the inequalities in this world that mean the decision to enrol in a malaria trial must be balanced with choices around household income and basic needs for one's family. I am grateful to every child and their guardian who took part in this trial; for their time, their trust and their commitment through 28 days of follow up.

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My two beloved children timed their arrival during the course of this thesis and it is dedicated to them. They have given me the perspective I needed and the continued motivation to focus my career on questions that matter. They both attended malaria conferences before they could sit up. They remind me to value the present moment and to wonder at our natural world.

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Abbreviations

ACT	artemisinin-based combination therapy
AL	artemether-lumefantrine
AUC	area under the plasma concentration-time curve
C_{max}	the maximum peak plasma concentration
CL/f	the mean oral clearance
Day 0-28	Day 0-28 after enrolment in the trial
dL	decilitre
DNA	deoxyribonucleic acid
DP	dihydroartemisinin-piperaquine
DSMB	data safety monitoring board
G6PD	glucose-6-phosphate dehydrogenase
Hb	haemoglobin
HIV	human immunodeficiency virus
MDA	mass drug administration
mRNA	messenger ribonucleic acid
PCR	Polymerase Chain Reaction
PK	pharmacokinetic
QT-NASBA	quantitative real time nucleic acid sequence-based analysis
$t_{1/2}$	elimination half life
WHO	World Health Organization

Glossary

C_{max}	The maximum peak plasma concentration: the maximum concentration of a drug in the blood after the drug has been administered and before the administration of a second dose
CL/f	The mean oral clearance: total clearance of a drug from the plasma after oral administration, measured in volume/ time
$t_{1/2}$	Elimination half-life: the time taken for the plasma concentration of a drug to be reduced by 50%

1 Introduction

1.1 Malaria, elimination, and the need for additional interventions

Malaria kills an estimated 435 000 people per year (1). The majority of these deaths are in African children, due to infection with *Plasmodium falciparum*. Considerable global morbidity is attributable to the complications of infection, including anaemia(2) , poor pregnancy outcomes (3), and, following severe cerebral malaria, cognitive and neurological impairment (4, 5). In any given epidemiological setting, infection is most prevalent in the poorest populations due to conditions that increase contact with the Anopheles mosquito vector, such as exposure-prone housing and working conditions, and due to compromised access to preventative healthcare and treatment (6). Endemic countries experience adverse financial and development consequences due to the burden of infection on the population (7, 8).

The last century saw the first collaborative effort to achieve global eradication of malaria. The World Health Organization (WHO) Malaria Eradication Programme in 1955-1969 co-ordinated multilateral control activities focussed primarily on vector control and widespread distribution of the anti-malarial drug chloroquine. The extent of global malaria endemicity was reduced significantly in regions with low, unstable transmission, but countries with the highest levels of malaria transmission and with the poorest health infrastructure, particularly in sub-Saharan Africa, received no deployment of interventions (9). In the ensuing two decades, these regions saw a rise in malaria-attributable deaths and global malaria interventions shifted focus to the control rather than the eradication of malaria and had relatively little impact (10). Important contributors were the widespread emergence of insecticide-resistant mosquitoes, and of chloroquine-resistant malaria parasites. In the last decade, strong and productive collaboration between committed funders, researchers and international organisations launched a new drive to tackle malaria as a public health problem. A bold renewed ambition of malaria eradication was declared by Bill and Melinda Gates in October 2007 (11) . This was

endorsed by the WHO in 2008 with a new Global Malaria Action Plan, calling for the elimination of malaria as a public health problem (12, 13) . Focussed distribution of insecticide treated bed nets, indoor residual spraying of households with insecticide, prompt and effective anti-malarial treatment, and intermittent preventive treatment of malaria during pregnancy (IPTp) has been accompanied by a significant fall in malaria deaths in all regions, particularly in African children (14). Several countries have reached “pre-elimination” and “elimination” status (1). Even in countries with the highest levels of transmission, there have been some significant and sustained gains following targeted malaria interventions (15, 16).

Malaria elimination requires that the number of infectious mosquito bites per person per year is reduced to zero in a defined geographic area (17). To achieve this, local transmission must be interrupted and maintained as such, and the importation of malaria into the area must be monitored and managed actively (18).

In the last five years, the estimated number of deaths from malaria globally has fallen by 17.8% (28% in children aged under 5 years) (1). A powerful analysis of field surveys and intervention coverage found that between 2000 and 2015, the prevalence of *Plasmodium falciparum* infection halved in endemic African countries (19). These massive gains imply the success of existing strategies; significant attribution has been given to the increased distribution of insecticide-treated bednets (19).

The WHO proposed a technical strategy for tackling malaria between 2016 and 2030 defining the target of eliminating malaria in 10 out of the 91 malaria-endemic countries by 2020 and in a further 25 countries by 2030 (13) . Whilst elimination is on track in some settings, the majority of endemic countries fall short of the indicator of reducing malaria case incidence and mortality by 40% by 2020 (1, 20). The falling trend in the annual global number of malaria deaths stalled between 2015-2016 and there was an increase in the annual number of malaria

cases, 90% of which were in the African region (21). In 10 sub-Saharan African countries and in India, the number of malaria cases increased in 2016, and again in 2017 (1).

Drug resistance in mosquitoes and in malaria parasites represents a major threat to the effectiveness of existing elimination efforts (13). Originating in Southeast Asia, resistance to the most powerful and effective antimalarials, artemisinin-based combination therapies (ACTs), is now well-documented (22). The magnitude of the role that antimalarial drug resistance, as opposed to insecticide resistance, plays in the number of malaria deaths needs to be understood. There is substantial evidence that counterfeit and substandard antimalarial drugs are responsible for malaria deaths (23, 24). Mathematical models predict that innovative implementation strategies are needed to bring transmission to elimination levels (25, 26).

Whilst vector control and case-based treatment have demonstrable impact (27-29), the reduction of transmission to zero requires that parasites are cleared from all reservoirs of infection. Case-based treatment targets symptomatic infections, but evidence points to a significant contribution of asymptomatic infection to ongoing transmission (30, 31). Prior to this thesis, relatively limited emphasis had been placed on targeting the transmission stages of the parasite and the role such a strategy might have in decreasing the time to elimination. The focus of this work is on blocking transmission of *Plasmodium falciparum* malaria infection from humans to mosquitoes, with an intervention that has not hitherto been adopted widely, namely, drug therapy targeted against the gametocyte, the sexual stage of the parasite.

1.2 Literature review

1.2.1 A focus on gametocytes and gametocytocidal therapy

1.2.1.1 *Role of the gametocyte in malaria transmission*

Theoretically, effective clearance of gametocytes from the human population would interrupt the malaria transmission cycle definitively. Targeting the gametocyte, or the interruption of gametocytogenesis, promises to be an important element of malaria elimination programmes (32, 33).

The mortality and morbidity associated with *Plasmodium falciparum* infection is due to the effects of the asexual stage of the parasites on the human red blood cell, reviewed in (34). As the parasite multiplies and proliferates within red blood cells, conformational changes in the red cell membrane render it rigid and sticky (35) resulting ultimately in cell rupture. Infected red cells bind to the vascular endothelium and to the placenta (in pregnant women). This adhesion results in further harmful effects on the host, such as cytokine release and microvascular pathology. Uninfected red cells are also reduced by removal in the spleen. Anaemia, inflammatory cell recruitment and organ damage result, proving fatal if untreated and unchecked by the host immune system (36).

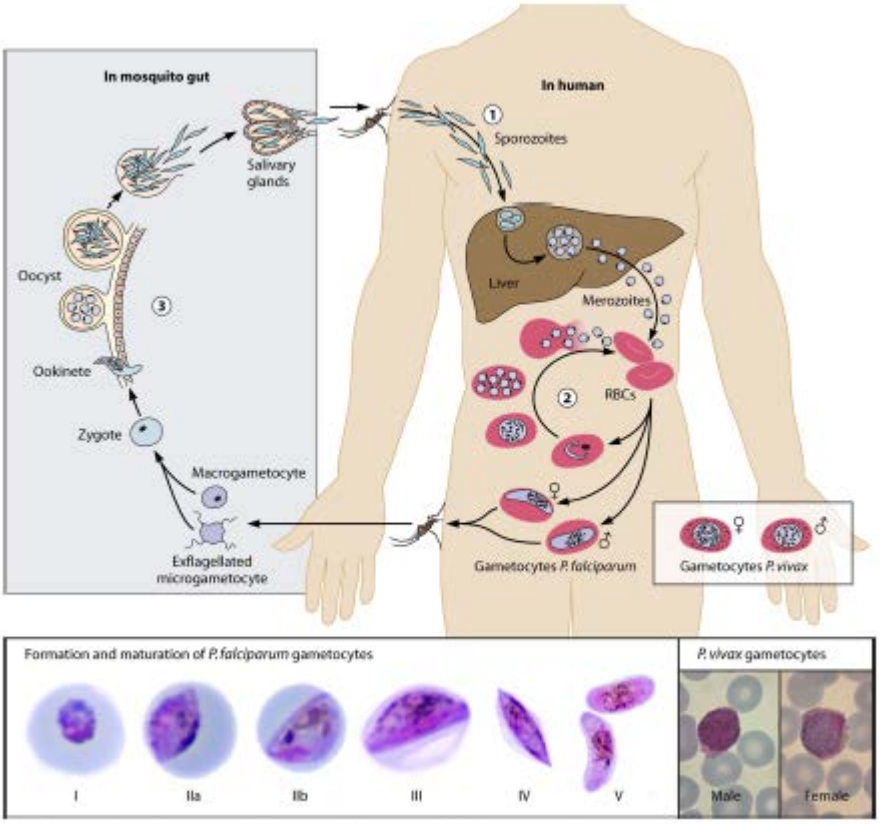


Figure 1-1 The lifecycle of *Plasmodium falciparum*: the role of the gametocyte.

A relatively small number of erythrocytic merozoites differentiate into the sexual forms of the parasite, the male and female gametocytes, rather than undergoing asexual reproduction in the human host. Gametocytes develop through five morphological stages within the haematopoietic system until they reach maturity (stage 5 gametocytes) and re-enter the peripheral circulation, where they are infectious to biting female anopheline mosquitoes. In the mosquito midgut, the parasites undergo sexual reproduction, forming an oocyst. Upon maturity, the oocyst ruptures and releases sporozoites which migrate to the mosquito salivary glands, enabling onward transmission to humans. From Bousema Drakeley Clinical Microbiology Reviews 2011 (37).

Early on in the course of human infection a small number of parasites differentiate sexually into male and female gametocytes (reviewed in Sinden 1983 (38)), (Figure 1-1). Gametocytes are inert and harmless to the human host. They sequester in the spleen and haematopoietic system where they mature through five stages of development and the mature stage 5

gametocytes are released in the peripheral blood (reviewed in Nixon 2016 (39)). Gametocytes outlive the harmful asexual stages, going on to circulate in the human host for days and even months after cure from clinical infection (40, 41) .

Theoretically, only two gametocytes (one of each sex) must be ingested per blood meal to effect successful transmission. Concentrations as low as 1 gametocyte per 5000 leucocytes have had documented infectivity to mosquitoes (42). The likelihood of human to mosquito transmission has been found to correlate with the density of gametocytaemia (43, 44). However, there is much observed non-linearity in this relationship (41, 45, 46). This has been explained by many factors, including the influence of antimalarial drugs (47, 48); human host-derived inhibitory mechanisms such as acquired transmission-blocking antibody responses to antigens on the gametocyte surface (49, 50) (leading the way to the development of malaria transmission-blocking vaccines (51)); the immune defence of the mosquito (52, 53); and factors intrinsic to the gametocyte itself, such as the ratio of male to female gametocytes and their longevity. To summarise, even gametocyte densities at, and below, the lower limit of detection are able to infect mosquitoes (46, 54) so interventions designed to clear them must be designed with this in mind. Although some countries, such as Sri Lanka (55), have seen success in malaria elimination principally by targeting a reduction in symptomatic cases interventions that only target infections of high enough density to produce symptomatic infections might not sufficiently interrupt human to mosquito transmission in countries with a large proportion of low density infections (56). In settings where low density infections are highly prevalent, case-based treatment, rather than broader campaigns to treat people with asymptomatic infections, may have reduced impact.

1.2.1.2 *Persistence of gametocytes post standard antimalarial drug interventions*

Many antimalarial drugs have activity against the sequestered early stage 1-4 gametocytes, reducing the number of parasites that will go on to become infectious after treatment (32, 47). Indeed, the introduction of ACTs saw reductions in transmission due in part to their rapid and highly-effective schizontocidal activity and also to their effect on early stage gametocytes (57). However, at the time of treatment, the majority of patients will have been infected for long enough to have developed a significant gametocytaemia (37) and it is these already-circulating mature stage 5 gametocytes that are responsible for transmission to the mosquito vector. Numerous studies have documented the persistence of mature gametocytes after

antimalarial treatment, including non-ACTs (58-61) and ACTs(62-65), enabling successful transmission to mosquitoes (37, 66).

Currently, the only drug class with *in vivo* activity against mature stage 5 gametocytes is the 8-aminoquinolines, and primaquine is the only drug in this class that is available and licensed widely (reviewed in White 2013 (67)).

1.2.1.3 *Primaquine's transmission-blocking activity*

1.2.1.3.1 Primaquine's gametocytocidal action

The 8-aminoquinolines were first developed in 1931 having primary action as antimalarials and antiseptics (68). The drug class was derived from the first synthetic antimalarial, methylene blue. Primaquine, 8-(4-amino-1-methyl-butylamino)-6-methoxyquinoline, was developed by the US Army and scientists at Columbia University in the 1940s for the purpose of radical cure and prevention of relapse of *Plasmodium vivax* infection (i.e., as a terminal prophylactic) in troops deployed in Southeast Asia and to prevent the importation of malaria when they returned home (69-71). It succeeded its parent compound, pamaquine (also known as plasmochin, plasmocide); the use of pamaquine was discontinued due to unacceptable levels of haemolysis and gastrointestinal toxicity (72, 73). Primaquine has sporozoiticidal activity and is recommended as an effective primary prophylactic agent for all species of malaria (74). There is some evidence that the drug may render hepatocytes non-receptive to sporozoites (75). Its effect against the asexual stages in the blood is unacceptably weak for use as a schizontocide for treatment (76). It was the demonstration that a single dose was effectively gametocytocidal (77-79) that led to recognition that primaquine has important public health potential to reduce transmission of *Plasmodium falciparum* parasites, regardless of their sensitivity to schizontocidal drugs (80). Evaluation of this potential is of particular

importance today, in the face of emerging parasite resistance to artemisinin derivatives in Southeast Asia, threatening the effectiveness of the most potent antimalarial drugs we have available.

Historical studies, conducted shortly after primaquine's development, measured primaquine's transmission-blocking efficacy at the level of the individual, at the level of the mosquito and at the level of the population. Primaquine administered to individuals reduced the gametocyte count after treatment (77, 78, 81). To assess the effect of primaquine at the level of the mosquito, blood samples taken from malaria-infected individuals were fed to laboratory-reared mosquitoes to assess mosquito infectivity. The likelihood of the development of oocysts in the mosquito midgut, i.e., successful transmission and sporogony, was reduced when the individual was treated with primaquine (77, 78, 81, 82). Only one historical study assessed the effect of primaquine administration on population level malaria transmission, but there was no control arm. Clyde conducted a mass administration of a primaquine-amodiaquine drug combination in sequential weekly, fortnightly and monthly rounds over a duration of ten months to three distinct populations of over 5000 individuals in eastern Tanzania (83). In addition to gametocyte prevalence, two additional measures were assessed to indicate ongoing transmission of the parasite beyond the treated individual: the mosquito sporozoite rate and the population asexual parasite rate. High population coverage was achieved (over 93%) and both sporozoite rates and population parasite rates reduced significantly with weekly and fortnightly treatment. However, monthly treatment intervals were much less effective, seeing a resurgence of parasitaemia prior to sequential doses. The author noted that population coverage was an important limiting factor to transmission interruption, as has been borne out by more recent modelling studies (84).

1.2.1.4 *Role of gametocytocidal drugs in malaria elimination prior to this work*

1.2.1.4.1 WHO guidelines prior to this work

In recognition of the documented efficacy of primaquine as a gametocytocidal drug, decades of WHO malaria treatment guidelines have incorporated recommendations for primaquine use to block malaria transmission. In 1973, WHO guidelines advocated the use of single dose primaquine at 0.75mg/kg (45mg adult dose) to block transmission, asserting that this dose was well-tolerated and there was no need to screen for G6PD deficiency prior to its use (80) . However, the setting for its use and the method of deployment was not stipulated. In 2001, the WHO Roll Back Malaria report advised the administration of 0.75mg/kg of primaquine to block malaria in areas of low to moderate transmission (85). The advice was to administer the drug after the patient had stabilized and again, that the dose was well-tolerated and that prior testing for G6PD deficiency was not recommended. In 2008, the WHO's Malaria Control and Elimination guidelines stated that the effect of ACTs on gametocytes was incomplete, so they should be combined with primaquine to block transmission more effectively (12). Again, there was no detail as to the timing or optimal setting for this treatment. After this thesis started, in 2010, WHO Malaria Treatment Guidelines advised primaquine as an addition to ACT as a component of a pre-elimination or elimination programme (86). Despite these repeated recommendations for primaquine as a malaria control tool, relatively few programmes incorporated its use, none of which were in Africa. This was largely due to the perceived risks associated with its use (reviewed in Ashley 2014 (87)).

1.2.2 Risks of primaquine

1.2.2.1 *Gastrointestinal symptoms*

At therapeutic doses, primaquine causes gastrointestinal side effects (abdominal pain, cramps, mild diarrhoea) when taken on an empty stomach. Taking primaquine with food, however, significantly reduces this effect, as well as increasing its bioavailability (88).

1.2.2.2 *Methaemoglobinaemia*

Methaemoglobin is produced by the oxidation of oxyhaemoglobin when iron is oxidised from Fe^{2+} to Fe^{3+}. This is a continuous process and it is regulated by the reducing nicotine adenine dinucleotide (NADH) system. Under oxidant stress, excessive methaemoglobin is formed. Primaquine is an oxidant that causes a predictable drug-induced methaemoglobinaemia. During a 14-day course of daily primaquine (15mg) for radical cure of *Plasmodium vivax* infection, typically less than 5% of total haemoglobin is methaemoglobin, and rarely greater than 12% (89). Symptoms are rare below levels of 15-20% (90) . In individuals with G6PD deficiency and NADH methaemoglobin reductase deficiency, excessive methaemoglobinaemia can occur (91). This can eventually reduce oxygen delivery to the tissues causing cyanosis, and at high levels, fatigue, dyspnoea, nausea and tachycardia.

1.2.2.3 *G6PD deficiency-related haemolysis*

Soon after the drug was developed, it became clear that certain individuals were 'primaquine sensitive'; they experienced haemolysis after exposure to primaquine (92, 93). The suspicion that an enzyme deficiency was the underlying cause (94) led to the elucidation of the underlying biochemistry, haematology and, subsequently, genetics of G6PD deficiency (95-97) .

In G6PD deficient individuals, the risk of haemolysis is well-documented following a 14 day course of primaquine for radical cure of *Plasmodium vivax* malaria (98, 99) . There are also case reports of severe haemolysis after administration of single dose primaquine. For example, cases of severe haemolysis and black urine have been reported in Vanuatu following a single dose of 45mg of primaquine (100) . In Tanzania, where the prevalent A- variant of G6PD deficiency is typically associated with mild deficiency, a child was found to have severe haemolysis after a single dose of primaquine (101) .

Primaquine is distributed to the tissues rapidly and undergoes hepatic metabolism. The toxicity of primaquine is due to one or more of its metabolites; the responsible compound has not yet been identified (102). The functional biochemistry of primaquine metabolites, reviewed by Vale in 2009, is poorly understood (103). The carboxyprimaquine and 5-hydroxyprimaquine metabolites have been proposed as candidate haematotoxic molecules (104, 105). Chemical instability hampers the investigation of the full range of metabolites. Primaquine is chiral and is usually produced in a racemic form (both L- and D- isomers present). Studies on stereo-selectivity indicate that the different enantiomers have different safety profiles and further work may produce products with reduced toxicity (106).

Haematological toxicity has limited the widespread use of primaquine. This has led, in recent years, to a proliferation of searches for safer new drugs with gametocytocidal activity (107, 108), with ensuing concepts and programmes for drug development (109). However, alternatives to primaquine are currently not readily available for deployment. The indication from two small studies from the 1960s that primaquine-induced haemolysis is dose-dependent (110, 111) led to the hypothesis for this thesis.

Now that low-dose primaquine is recommended by the WHO as an adjunct to standard antimalarial therapy in malaria elimination and containment programmes (in addition to the well-established recommendation for radical cure of *Plasmodium vivax* malaria), it is set to be deployed more widely in malaria endemic regions (112). The risk of primaquine-induced haemolysis has demanded further and urgent exploration. The distribution of malaria endemicity roughly mirrors the prevalence of G6PD deficiency (Section 1.2.3.4). There is some speculation as to whether this is driven by vivax or falciparum malaria infection. It is crucial, therefore, that we understand the risk of drug-induced haemolysis both at the individual level and at the population level for primaquine administered at an efficacious dose for transmission-blocking.

The following section reviews the background pathophysiology, epidemiology and available diagnostics for G6PD deficiency that have implications for risk and safety in primaquine deployment.

1.2.3 G6PD deficiency

1.2.3.1 *What does the G6PD enzyme do?*

G6PD is expressed in almost all human cells, including red blood cells, and is essential to their functioning (113). It catalyses the first step in the pentose phosphate pathway of carbohydrate metabolism, a series of reactions that ultimately results in the production of the reducing molecule NADPH. This confers cells with protection from potential oxidative damage. The enzyme is encoded by an X-linked gene which is highly polymorphic. More than 186 mutations have been described to date (114, 115), leading to phenotypes, varying in biochemistry and clinical manifestation (116-119). Despite this extensive polymorphism, the vast majority of mutations are single point substitutions and all are in the coding region of the gene, supporting the assertion that a baseline level of G6PD expression is necessary for survival (120).

1.2.3.2 *Ethical reflections on early work on G6PD deficiency*

A significant part of the work that led to the first definitions of G6PD deficiency, and that has been used subsequently to inform contemporary primaquine use, was based on by experiments conducted on inmates of the US Stateville Penitentiary, Joliet, Illinois (121-124). This was through a collaboration of the US Army Malaria Research Programme and the University of Chicago in the 1940s (93). These experiments were exhibited during the 1947 Doctor's Trial that led to the development of the Nuremburg Code of ethics of human subjects research, now superseded by the Declaration of Helsinki (125). Endorsed at the time of the trial, their ethical basis has been criticised subsequently (121, 126).

1.2.3.3 *What are the physiological effects of G6PD deficiency?*

1.2.3.3.1 Haematological effects of G6PD deficiency

G6PD deficient individuals exhibit varying degrees of fragility of the enzyme product, depending on the specific mutation they carry (127). The extent of enzyme fragility translates to a risk of haemolysis (97, 119). Red blood cells are particularly affected by G6PD deficiency because, having lost their nucleus and key organelles during development (a crucial step that facilitates their unique role in transporting oxygen), they are unable to produce enough functioning G6PD enzyme as they age. Furthermore, G6PD enzyme activity decreases as they age (94). They are unable to compensate because they lack mitochondria to produce NADPH from alternative pathways. Low levels of functional G6PD enzyme renders red blood cells vulnerable to haemolysis under oxidative stress, which triggers a reticulocytosis to buffer against the oxidative challenge (110).

The oxidative products of foods (archetypally, fava beans (128)), infections (129), and drugs (92, 110, 116, 130) such as primaquine, can trigger haemolysis, reviewed in (118, 131). The risk of haemolysis is governed by factors that determine the exposure to the drug, or other trigger factor, such as drug dose (92, 110), drug metabolism and drug-drug interaction (88, 132) or inter-current infections (133, 134). Additional, extrinsic factors affecting the pharmacokinetics of primaquine are summarised in Table 1-1.

Rare mutations producing the most severe deficiency cause a chronic haemolysis with no exogenous trigger, known as congenital non-spherocytic haemolytic anaemia, more analogous to severe thalassaemia (129, 135). These mutations are considered to be sporadic and independent in their origin, compared to the inherited, conserved milder variants that cluster in malaria-endemic regions (136, 137).

The WHO classification of G6PD deficiency according to phenotype is presented in table 5-1. Whilst male hemizygotes (their sole X chromosome carries the deficient gene) and female homozygotes have a fairly predictable phenotype, female heterozygotes can exhibit a range of phenotypes. This is attributed to lyonisation, whereby one of the two X chromosomes is randomly inactivated in each cell (138). The implications of this for the diagnosis of G6PD deficiency and for primaquine deployment are discussed later in this chapter (Sections 1.2.3.6).

Table 1-1 WHO classification of G6PD deficiency. Adapted from WHO (116) and Capellini *et. al.,* 2008 (118)

Category	Description	Residual G6PD enzyme function	Common Variants
Class I	Severely deficient and associated with chronic non-spherocytic haemolytic anaemia	Minimal	(Very rare) Zacatecas (139), Hamburg (140), Veracruz (139), Yucatan (139)
Class II	Severely deficient and associated with acute haemolytic anaemia	1-10%	Mediterranean (141), Santamaria (142), Viangchan (143), Jammu (144), Seattle (145)
Class III	Moderate to mild deficiency	10-50%	A- (146), Mahidol (147)
Class IV	Normal activity	60-150%	A, B (146)
Class V	Increased activity	>150%	

1.2.3.3.2 Clinical effects of G6PD deficiency

The clinical presentation ranges from self-limiting haemolysis (110) to life threatening effects on the kidneys resulting in haemoglobinuria and acute renal failure (99, 148). Neonatal jaundice may be self-limiting or, in severe cases, result in kernicterus (149). Severe haemolysis is characterised by symptoms of fatigue and back pain, and signs of anaemia,

jaundice and haematuria (dark, blood-stained urine) (reviewed in (118, 129). The resultant anaemia is accompanied by an unconjugated bilirubinaemia, raised lactose dehydrogenase, and reticulocytosis (150). Management of haemolysis depends on the severity and a key intervention is avoiding or removing the trigger. Mild to moderate drug-induced haemolysis is typically transient and recovery ensues several days after stopping the drug. Severe haemolysis may require blood transfusion. In neonates, if ongoing haemolysis results in levels of unconjugated bilirubin above age thresholds (151), then phototherapy is given to prevent neurological damage. If levels are life-threatening, then exchange transfusion may be indicated.

1.2.3.4 *G6PD epidemiology/geodistribution*

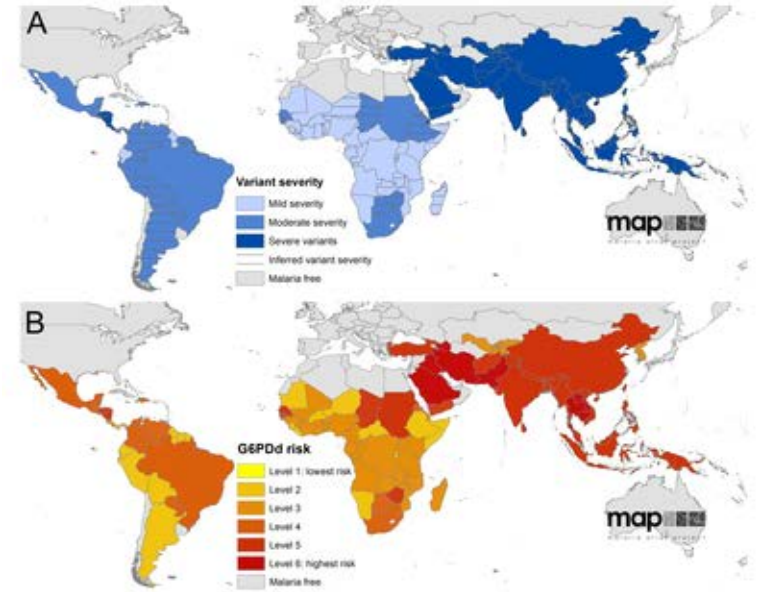
An estimated 400 million people are affected by G6PD deficiency globally (118). It is the most common enzyme deficiency worldwide and it is particularly conserved in malaria-endemic areas. In keeping with the hypothesis of Haldane in 1949 (152), that resistance to infectious disease drives natural selection in humans, there is solid evidence to suggest that G6PD deficiency affords protection against severe falciparum malaria (153, 154) *In vitro*, the growth of falciparum malaria parasites appears to be impaired in red blood cells with reduced G6PD function (155, 156), and they are more readily phagocytosed (157). At population level, the risk of severe falciparum malaria was significantly reduced in male hemizygotes in a hospital-based case-control study in Mali (158) and in both male hemizygotes and female heterozygotes in hospital- and community-based case-control studies in Kenya and The Gambia (159, 160). A large prospective cohort study in Uganda found a significantly reduced incidence of malaria episodes in phenotypically G6PD deficient females but not males (161, 162), reflecting risk in a more representative community-based sample than hospital-based surveys of people with malaria. Individuals with malaria are haemolysing and therefore, have a higher mean G6PD enzyme level in the surviving red blood cells. Using G6PD genotyping to estimate the level of enzyme activity, a large multi-centre case-control study found that the

type of clinical manifestation of severe malaria was associated with the extent of G6PD enzyme deficiency. Decreasing levels of enzyme activity (more severe deficiency) were associated with a higher risk of severe malarial anaemia but a lower risk of cerebral malaria (163). G6PD deficiency was associated with significantly reduced risk of *Plasmodium vivax* malaria infection in an Afghan population (164) and with reduced *Plasmodium vivax* parasite density in Thailand (165).

Global prevalence estimates indicate that the highest population frequencies of G6PD deficiency are in sub-Saharan Africa with hotspots also in the Mediterranean, the Arabian Peninsula and in parts of South and Southeast Asia and the central and southern Pacific islands (116, 166). Moderate levels are found in the Americas (167, 168). In regions where the G6PD map does not correspond to malaria prevalence, this has been attributed to relatively recent migration from malaria-endemic areas or successful regional malaria eradication (118).

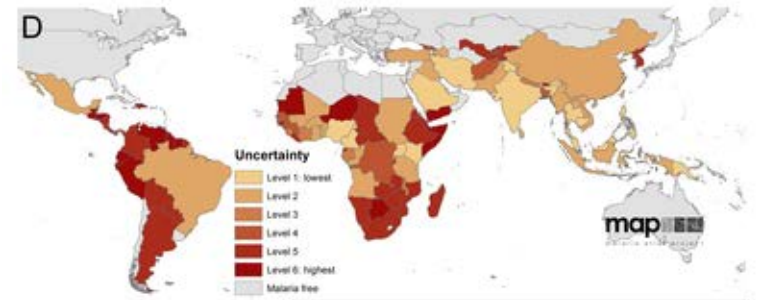
Recently, a novel approach to mapping the global distribution of G6PD deficiency was applied by Howes and colleagues (167). Data from 1734 surveys of phenotypic enzyme function was screened for quality and sub-national geotatistical mapping methods were used to generate global- and national-level maps of the allele frequency of G6PD deficiency. The investigators incorporated both estimates of the certainty of the data and population-weighting. The highest prevalences of enzyme deficiency were found in sub-Saharan Africa (with relative sparing of the Horn of Africa and parts of southern Africa). Whilst the prevalence was lower in Asia, when it was weighted by population density, the highest burdens were found in this region, particularly in India and China. Howes overlaid prevalence data with scores of the severity of G6PD deficiency from data on the variants found in geographical surveys to produce a map that highlights the overall risk from G6PD deficiency, and the implied risk of haemolysis (Figure 1-2). The highest risk regions were in west Asia and the Arabian Peninsula and it was high across the whole of Asia. This distinction, between risk and prevalence, must

be taken into consideration in primaquine deployment programmes. In summary, whilst the A- variant is highly prevalent in sub-Saharan Africa, the mild severity of the enzyme deficiency (10-20% residual enzyme function) could be expected to present a lower risk of haemolysis in the context of treatment with primaquine compared to more severe variants.



C G6PDd risk index

		Variant severity		
		Class III only	Class II uncommon	Class II common
National G6PDd prevalence	Rare: ≤1%	Level 1 (n = 1)	Level 2 (n = 7)	Level 3 (n = 7)
	Common: >1 - 10%	Level 2 (n = 13)	Level 4 (n = 15)	Level 5 (n = 20)
	High: >10%	Level 3 (n = 20)	Level 5 (n = 5)	Level 6 (n = 11)



E Uncertainty index

		Reliability of severity score		
		Low uncertainty	Medium uncertainty	High uncertainty
Prevalence uncertainty (IQR/Median)	Low: ≤ 50%	Level 1 (n = 13)	Level 2 (n = 4)	Level 3 (n = 0)
	Medium: >50 - 100%	Level 2 (n = 19)	Level 4 (n =6)	Level 5 (n = 9)
	High: >100%	Level 3 (n = 7)	Level 5 (n = 21)	Level 6 (n = 20)

Figure 1-2 Severity risk from G6PD deficiency. From Howes *et. al.* (167)

A: G6PD variant severity score per country (ratio of class II to class III variants). B: G6PD deficiency risk index (severity of variants, from A, and prevalence of G6PD deficiency). C: Scoring matrix for maps A and B. D: uncertainty level of data analysis for severity score and risk index per country. E: matrix for uncertainty index for severity and prevalence (detailed in Howes et. al. (167))

1.2.3.5 *G6PD deficiency in Uganda*

There is marked geospatial heterogeneity in the prevalence of G6PD deficiency within Uganda and this appears to vary with the regional risk of malaria (169). Prevalences vary depending on the method of analysis (162) (see section 5.5). A recent cross sectional survey in South western Uganda screened 631 asymptomatic children aged between 6 and 59 months of age for G6PD deficiency and found low but varying prevalence depending on the assay used (170). Applying a <60% threshold of activity, a quantitative enzyme activity assay (Trinity Biotech ® G6PDH test, Ireland) found 6.8% of children with mild or moderate deficiency (none with severe deficiency), whilst a qualitative rapid diagnostic test (CareStart^{TM}) identified 8.6% of children as deficient. In Tororo district, in the East, enzymatic testing of children in a community cohort study found 19.7% of children had mild or moderate G6PD deficiency (60% activity cut-off) (171). Gold standard genotyping found the prevalence of the G202A mutation to be 6.8% in this population. A household level survey conducted in 1344 individuals distributed across three districts with varying malaria endemicity found prevalences of G6PD A- variant (G202A mutation) ranging from 8% in the low endemic setting (Kanungu district) to 29% in the high endemic setting (Tororo) (169). In Walukuba, Jinja, the study site for this thesis, this household survey found a genotypic prevalence of 18%. A longitudinal cohort study of children in the neighbouring district of Iganga, the prevalence was comparable, 22.7% in a 1-year cohort study (172) and 20.4% in a birth cohort (173).

1.2.3.6 *The challenge of testing for G6PD deficiency*

The exact mechanism for primaquine-induced haemolysis is still unknown. Tests for an individual's risk of haemolysis are based on the proxy measure of the level of residual G6PD enzyme function (phenotypic tests) or on their genotype (genotypic tests).

To accurately predict the risk of exposure to a drug like primaquine, we need to answer a key question: how does a given level of G6PD enzyme activity correlate with the risk and severity

of haemolysis after primaquine administration both at individual level, and at population level? This remains to be clearly delineated, leaving, on one hand, the risk of causing harm to individuals with a low threshold for primaquine-induced haemolysis and on the other hand, the risk of reducing the maximal impact of primaquine on interrupting transmission by omitting individuals from the intervention (174, 175).

1.2.3.6.1 Available tests for G6PD deficiency

1.2.3.6.1.1 Phenotypic tests of enzyme function: Qualitative Quantitative

Quantitative assessment of G6PD enzyme activity enables classification of the degree of enzyme deficiency, and this can be interpreted using the WHO classification of severity (Table 1-1). A shortcoming of the application of this classification for clinical use is that, although the severity categories relate to a specific range of enzyme function, they are not calibrated to the risk of haemolysis induced by primaquine or any another precipitant.

The gold standard method for quantitative phenotypic assessment is laboratory-based ultraviolet spectrophotometry. Accurate results depend on preservation of functioning enzyme levels from the point of blood sampling through to the point of running the assay. This applies to both the test samples and to the biological controls (171). G6PD enzyme degrades at room temperature and is preserved for two weeks at 4-8 degrees (176). Freezing allows longer-term storage, but the enzyme degrades on thawing (171). The haemolytic status of the test recipient can affect assay results. During acute haemolysis old erythrocytes are removed selectively. The remaining young reticulocytes have less-fragile G6PD enzyme, so higher functioning activity levels (177). This can lead, potentially, to false normal classification. To avoid misclassification, the WHO recommends an adjustment calculation depending on haematological status (178). Typically, a normalisation adjustment is made for haemoglobin level, but when anaemia is caused by a haemoglobinopathy, red cell count normalisation is recommended instead, to avoid over-estimation of G6PD enzyme activity (179). Sampling

from venous versus finger-prick capillary blood does not appear to affect measured G6PD activity, despite the slight difference in haemoglobin concentration and red cell count anticipated by the two methods (180). Females may be under-diagnosed with phenotypic tests. The linkage of the G6PD gene to the X chromosome was established in 1961 (181). In females, each cell expresses only one copy of the X chromosome and this process, lyonisation, occurs at random (182), the classic example being the mosaicked colouring of the coat of the tortoiseshell cat. Hence, homozygous females have a predictable G6PD phenotype, but female heterozygotes have a highly variable extent of gene expression (129, 183) and they may be under-diagnosed when using conventional enzymatic tests rather than molecular diagnostics (97, 131).

A range of field-based methods have been developed to quantify enzyme activity in resource-limited settings and increasingly, recommendations are being designed to minimise potential challenges and to standardise test evaluation (184).

Point-of-care tests are becoming available, designed to simplify requirements for laboratory training and facilities (185, 186) and there is indication that they may be cost-effective (187). These tests are largely qualitative; with cut off values that vary widely, from 10-60% of residual enzyme function (137, 174, 185). The WHO recently published prequalification criteria for qualitative tests (188). These require that tests determine G6PD status as a percentage of the adjusted male median enzyme activity for a population: the “normal” cut off is defined as >30% in males and >80% in females (189).

1.2.3.6.1.2 Genotypic tests

A given G6PD genetic variant is loosely associated with a clinical phenotype, as illustrated in Table 1-1 (Section 1.2.3.3.1) for the most prevalent variants. However, there are documented cases where this is not consistent. For example, whilst G6PD A- variant is usually associated with mild haemolysis, Shekalaghe *et al.* describe the case of a child who received single dose

primaquine during a mass drug administration resulting in severe haemolysis (101). In a household cohort study, Johnson *et al* found that 29% of males with reduced enzyme function had wild type genotype for A- variant (162). A possible explanation is that these children have a genotype not yet characterised and not detected by the single nucleotide polymorphism (SNP) markers used for A- genotyping. Exploratory sequencing and screening for an extended range of SNPs and alternative genetic markers may reveal alternative genotypes prevalent in Africa that are associated with G6PD deficiency (163, 190). More than 400 biochemical variants have been defined, which far exceeds the number of molecular variants, approximately 187, that have been characterised (115). Furthermore, particular genotypes are found to be associated with a range of clinical presentations (119). A range of phenotypic severities have been documented in well-studied variants such as Mahidol (191), and A- variants (192). The risk of haemolysis for any given variant may have multiple determining factors. There is variability according to physiological status; immediately after an episode of haemolytic anaemia, a resistant period is documented in some genotypes, during which further oxidative insult does not produce any worsening in haemolysis, such as is seen in A- variant but not in Mediterranean (97). To summarise, genotyping alone does not give a reliable indication of an individual's capacity for haemolysis at a given instance.

Molecular tests are typically unsuited to field settings because of the high requirement for resources, including technical equipment and consumables stored at constant temperature conditions, with reliable electricity, and highly-trained operating personnel.

The thesis presented an opportunity to provide samples for the development of a novel high-throughput bioluminescence-based assay that enables the detection of multiple SNPs (20-50) without the use of gel electrophoresis. This assay still requires the resources for polymerase chain reaction, but it is an example of a methodology that might ultimately be transferable to the field (193).

1.2.4 Evidence-based study design

Prior to development of the protocol for this thesis, the evidence base was reviewed to inform the formulation of the research question (section 1.3 to 1.5) and the trial design (chapter 2). A large body of evidence for the use of single dose primaquine is now available. A series of Cochrane reviews (194-197) and the Worldwide Antimalarial Resistance Network (WWARN) (198) have kept track of the rapidly evolving research questions and data pool. However, this was not the case at the time this trial was conceived. Primaquine was recommended in guidelines, but the evidence base needed strengthening in order to empower policy makers to make informed decisions about its use.

This necessitated a process of thought as to what evidence gaps needed testing in a clinical trial and how such a trial should be structured. Superimposed was the need to produce a meaningful trial result in a timescale that that could enable its translation into policy rapidly, as, in several settings, primaquine use as a transmission-blocking agent was already in consideration (199).

It became apparent that a novel approach was needed to design a drug efficacy trial to assess transmission-blocking efficacy rather than asexual parasite clearance as per standard antimalarial drug efficacy trials. Although previous authors had studied the effect of primaquine on gametocytes, there was paucity of reference material for trial components such as optimal endpoints, sample size determinations, safety considerations, and the structure of follow-up procedures. Importantly, an emerging body of evidence was indicating that infections with very low sexual parasite densities (below the microscopic detection threshold) were infective to mosquitoes (54), so submicroscopic molecular detection methods needed consideration in trial design.

The process of informing study design is presented as a series of answers to questions in Chapter 2. Section 2.1.3 summarises the conclusions of this process, using the evidence that

was available at the time the trial objectives were formulated. In the discussion chapter (Section 5.2), the trial is put back into the context of the subsequent, contemporary evidence base.

1.2.5 Overall aim

To design, conduct and report a clinical trial to evaluate the efficacy and safety of lower doses of primaquine for the clearance of gametocytes in uncomplicated falciparum malaria in sub-Saharan Africa, compared to the reference WHO-recommended dose of 0.75mg/kg primaquine base.

1.2.6 Hypothesis

The trial hypothesis was that lower doses of primaquine given with ACT have a higher risk of adverse effects compared to ACT alone, and that they are not as efficacious as the WHO-recommended 0.75mg/kg dose for gametocyte clearance.

This hypothesis was tested with a four-arm clinical trial with a non-inferiority design to evaluate the efficacy, and with a superiority design to evaluate the safety, of the WHO dose (0.75mg/kg) and lower doses of primaquine for clearance of *Plasmodium falciparum* gametocytes in children in Uganda. The study was designed to include a novel pharmacokinetic analysis. The inclusion of an ACT-alone arm enabled testing of the hypothesis that primaquine adds no benefit for clearance of gametocytes compared to ACT alone and that primaquine is less safe than ACT alone.

1.2.7 Objectives

1.2.7.1 *General objective*

To evaluate the efficacy and safety of different doses of primaquine administered with standard antimalarial treatment, artemether lumefantrine (AL), to children in Uganda with uncomplicated malaria and with normal G6PD enzyme function, for the purpose of reducing *Plasmodium falciparum* gametocytes in the infected human host to prevent transmission of falciparum malaria to the *Anopheles* mosquito vector.

1.2.7.2 *Specific objectives*

1) To evaluate the efficacy of different doses of primaquine when administered with AL as measured by gametocyte prevalence and density

2) To evaluate the safety of different doses of primaquine when administered with AL as measured by change in mean haemoglobin, prevalence of severe anaemia (Hb <5g/dL), and evidence of black urine (haemoglobinuria; dipstick positive) or requirement for blood transfusion

3) To assess the safety of different doses of primaquine when administered with AL as measured by prevalence/ incidence of adverse events and tolerability

4) To obtain basic pharmacokinetic parameters for primaquine in the study population

5) To evaluate primaquine safety according to G6PD genotype and enzyme function for children who are misclassified by phenotypic G6PD testing

1.2.7.3 *Objectives for the thesis*

1.2.7.3.1 Design and implement a clinical trial

Funded as a clinician's research training fellowship, through the Wellcome Bloomsbury Clinical PhD Programme in International Health, the objective of the PhD was to design a clinical trial compliant with good clinical practice and regulatory body obligations, to attract an advisory panel of suitable expertise, to select an appropriate location and local collaborators for the trial and to implement it, manage it, control the budget and close the trial in a responsible manner.

1.2.7.3.2 Timely and accessible presentation of trial findings

Given the public health importance of baseline data on primaquine as a transmission-blocker, the reporting aim was to produce reports of the trial results in peer-reviewed journals. Prospective publication of the trial protocol was planned in order to uphold transparency and concept-sharing within the scientific community. This was important as, following the establishment of the Single Low-Dose Primaquine Working Group (Section 3.3.4.1), a range of future trials were planned in different settings (199).

2 Methods

2.1 RESEARCH PAPER 1: Publication of the trial methods

The trial methodology was published prospectively in a peer reviewed journal, BMJ Open 2012 (200). Sections 2.2 onwards describe the process of protocol development.

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RESEARCH PAPER COVER SHEET

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SECTION A – Student Details

Student ID Number	257918/RITD	**Title**	Dr
First Name(s)	Alice Chijioke		
Surname/Family Name	Eziefula		
Thesis Title	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	BMJ Open		
When was the work published?	25th February 2013		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	**No**	Was the work subject to academic peer review?	**Yes**

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Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I conceived and designed the study together with SGS, SY, NJW, TB and CD and I undertook logistical planning together with EW and MK. I organised the ethical applications, community sensitisation and study implementation and I wrote the manuscript together with TB and CD.

SECTION E

Student Signature	Chi Eziefula [redacted]
Date	18th September 2019

Supervisor Signature	
Date	23rd September 2019

BMJ open accessible medical research

Study protocol for a randomised controlled double-blinded trial of the dose-dependent efficacy and safety of primaquine for clearance of gametocytes in children with uncomplicated falciparum malaria in Uganda

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ABSTRACT

Objectives: For the purpose of blocking transmission of Plasmodium falciparum malaria from humans to mosquitoes, a single dose of primaquine is recommended by the WHO as an addition to artemisinin combination therapy. Primaquine clears gametocytes but causes dose-dependent haemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Evidence is needed to inform the optimal dosing of primaquine for malaria elimination programmes and for the purpose of interrupting the spread of artemisinin-resistant malaria. This study investigates the efficacy and safety of reducing doses of primaquine for clearance of gametocytes in participants with normal G6PD status. Methods and analysis: In this prospective, four-armed randomised placebo-controlled double-blinded trial, children aged 1–10 years, weighing over 10 kg, with haemoglobin ≥8 g/dl and uncomplicated P falciparum malaria are treated with artemether lumefantrine and randomised to receive a dose of primaquine (0.1, 0.4 or 0.75 mg base/kg) or placebo on the third day of treatment. Participants are followed up for 28 days. Gametocytaemia is measured by quantitative nucleic acid sequence-based analysis on days 0, 2, 3, 7, 10 and 14 with a primary endpoint of the number of days to gametocyte clearance in each treatment arm and secondarily the area under the curve of gametocyte density over time. Analysis is for non-inferiority of efficacy compared to the reference dose, 0.75 mg base/kg. Safety is assessed by pair-wise comparisons of the arithmetic mean (±SD) change in haemoglobin concentration per treatment arm and analysed for superiority to placebo and incidence of adverse events. Ethics and dissemination Approval was obtained from the ethical committees of Makerere University School of Medicine, the Ugandan National Council of Science and Technology and the London School of Hygiene and Tropical Medicine.

Results: These will be disseminated to inform malaria elimination policy, through peer-reviewed publication and academic presentations.

ARTICLE SUMMARY

Article focus

- Single-dose primaquine, administered together with artemisinin combination therapy, blocks transmission of Plasmodium falciparum malaria by clearing gametocytes.
- Primaquine, an 8-aminoquinoline, causes dose-dependent haemolysis in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Evidence is lacking on the safety and efficacy of lower doses of primaquine.
- This is the protocol of a dose-finding trial being conducted in eastern Uganda.

Key messages

- Dose-finding is a priority for the use of primaquine in malaria elimination programmes and to block the spread of artemisinin-resistant malaria.
- This trial is designed to investigate the efficacy and safety of reducing doses of primaquine for gametocytocidal action.
- This paper highlights the unique trial design issues that are relevant for investigating the efficacy and safety of antimalarials targeted against the sexual stages of malaria for blocking transmission rather than clinical cure.

Strengths and limitations of this study

- For ethical reasons, in this trial, dose-finding is conducted in children with normal G6PD status, but, ultimately, information is needed on the safety of lower doses in people with G6PD deficiency.
- This trial measures primaquine's transmission-blocking potential by assessing gametocyte clearance. Endpoints of mosquito transmission at multiple time points could be usefully assessed but on smaller numbers of individuals.

BACKGROUND

Sustained deployment of vector control measures and accessible, effective drug therapy has

reduced the transmission of Plasmodium falciparum in many endemic countries. However, further scaling-up of cur-rently available malaria control measures is unlikely to achieve malaria elimination in most settings.[1] Moreover, the emergence of resistance to artemisinin in Southeast Asia,[2 3] and the development of insecticide resistance and adaptive behaviour in the mosquito vector[4–6] present sig-nificant threats to the current trend of declining malaria burden. Malaria elimination initiatives and artemisinin-resistance containment strategies both require additional tools that are specifically aimed at reducing the transmis-sion of malarial parasites.[7 8]

Antimalarial drugs are designed primarily to target the asexual stages of the parasite that cause morbidity and mortality. The effect of antimalarial drugs on game-tocytes, the transmission stages, has for decades been seen as ancillary. P falciparum gametocytes undergo complex development that is characterised by five mor-phologically distinct stages of maturation.[9] The imma-ture gametocyte stages (I–IV) are sequestered in the reticuloendothelial system and bone marrow.[10–12] Mature stage V gametocytes typically appear approxi-mately 12 days after the onset of patent asexual blood-stream infection, and are the only gametocyte stage that circulates in the peripheral blood and is infective to biting female Anopheles mosquitoes.[13 14] The majority of antimalarial drugs, including artemisinins, lumefantrine and piperaquine, have some efficacy against immature gametocytes.[15 16] These drugs have the potential to reduce transmission at a population level because asexual parasites are cleared, preventing de novo devel-opment of gametocytes, and fewer of the immature gametocytes that are present upon initiation of treat-ment survive to maturity. However, the vast majority of symptomatic cases have measurable and transmissible levels of mature gametocytes at presentation.[17 18] These persist after treatment with all antimalarials that are cur-rently implemented as first-line treatment, including artemisinin combination therapy (ACT). Gametocytes that persist after ACT have repeatedly been shown to be infectious to mosquitoes.[17 19 20] This post-treatment gametocyte carriage frequently occurs at low densities, commonly below the microscopic threshold for detec-tion,[21 22] but is sufficiently high for efficient mosquito infection.[17 23]

The only class of drugs that are effective against mature P falciparum gametocytes is the 8-aminoquinolines. Primaquine is the most widely available drug in this class. The exact mechanism for this gametocytocidal activity is unknown, but it is probably dependent on oxidative damage to the intraerythrocytic parasite by primaquine metabolites.[24] Primaquine as a single dose of 0.75 mg base/kg added to standard ACT has superior gametocyto-cidal activity to ACT alone.[25–27] All doses of primaquine described hereafter refer to the dose of primaquine base per unit weight. There are indications that doses of primaquine lower than 0.75 mg/kg may be equally effica-cious. A Thai study showed that both 0.5 and 0.25 mg/kg of primaquine administered with ACT to adults infected with malaria effectively and indistinguishably reduced the proportion of mosquitoes that became infected after a blood meal.[28] In small numbers of adults, total doses of 30 mg and 15 mg have shown comparable efficacy to a 45 mg dose in reducing mosquito infection rates.[29 30]

The efficacy of primaquine when given as a single low dose is important in the light of concerns over the haematological safety of primaquine. There is conclusive evidence for primaquine-induced haemolysis in glucose-6-phosphate dehydrogenase (G6PD) deficient individuals.[31 32] G6PD deficient individuals are vulner-able to oxidative stress because their erythrocytes do not have alternative pathways for G6PD-dependent nicotina-mide adenine dinucleotide phosphate production, which is essential to maintain antioxidant defences. There is conflicting evidence on the risk of haemolysis after a single dose of primaquine. A single dose of 45 mg primaquine administered to a Vanuatan adult caused life-threatening haemolysis.[33] In G6PD-deficient Tanzanian children, the mean fall in haemoglobin after a single dose of 0.75 mg/kg primaquine was 2.5 g/dl (95% CI 1.2 to 3.8 g/dl), though no associated severe adverse events were recorded and haemolysis was transient.[34] On the other hand, primaquine was reported to be well tolerated when 0.75 mg/kg was given without prior G6PD testing in large studies in Myanmar, Sudan, Russia, Cambodia and China.[27 31 35 36]

Because primaquine-induced haemolysis is dose-dependent,[29] and because gametocytocidal efficacy may be retained with primaquine doses lower than 0.75 mg/kg, the WHO-recommended dose in its 2010 Guidelines for the Treatment of Malaria, dose-finding studies are needed urgently. This trial tests the hypoth-esis that lower doses of primaquine have a substantially lower risk of, or an absence of, adverse effects but that their gametocytocidal efficacy is retained.

METHODS AND ANALYSIS

Study design

The study is a prospective, randomised, parallel arm, placebo-controlled, double-blinded clinical trial of redu-cing doses of primaquine administered with artemether lumefantrine (AL) for the treatment of uncomplicated clinical P falciparum malaria infection in children aged 1–10 years of age. The study uses a non-inferiority design to evaluate the efficacy and a superiority design to evaluate the safety of 0.1 and 0.4 mg/kg primaquine compared with 0.75 mg/kg when added to AL.

Study objectives

1. To evaluate the efficacy of 0.1, 0.4 and 0.75 mg/kg primaquine when administered together with the fifth dose of AL as measured by gametocyte preva-lence and density.
2. To evaluate the safety of 0.1, 0.4 and 0.75 mg/kg primaquine when administered together with the

fifth dose of AL as measured by change in mean haemoglobin, prevalence of severe anaemia (haemoglobin <5 g/dl) and evidence of black urine (haemoglobinuria).

3. To assess the safety of different doses of 0.1, 0.4 and 0.75 mg/kg primaquine when administered together with the fifth dose of AL as measured by prevalence/incidence of adverse events and tolerability.

Participants and enrolment

The study is conducted at Walukuba Health Centre IV in Walukuba subcounty, Jinja district, in eastern Uganda. In this area, malaria transmission is year-round with two seasonal peaks. The entomological inoculation rate (EIR) was estimated at 7 infectious bites per person per year in Walukuba.[37] Study participants are recruited from children attending the Health Centre IV with sus-pected malaria (figure 1). Inclusion criteria are age 1–10 years, weight over 10 kg, fever (tympanic tempera-ture >38°C) or history of fever in the last 24 h, P falcip-arum mono-infection with a parasite density <5 00 000/ µl and normal G6PD enzyme function. Exclusion cri-teria are evidence of severe illness/danger signs, known allergy to study medications, haemoglobin <8 g/dl, started menstruation, pregnancy or breastfeeding, anti-malarials taken within the last 2 days, primaquine taken within the last 4 weeks and blood transfusion within the last 90 days.

The fluorescent spot test[38] is used for G6PD screen-ing. This test has a cut-off of approximately 20% enzyme function, below that, there is no fluorescence. The WHO classification defines severe G6PD deficiency as 10% enzyme function.[39]

Randomisation, blinding and intervention

After enrolment (day 0), participants are randomised to a treatment arm stratified by gender (figure 2). The study pharmacist selects sequential opaque envelopes (from either the male or the female pile). Each enve-lope contains a predetermined treatment assignment code. The study pharmacist is the only member of the clinic team not blinded to the treatment arm and is not involved in assessing patients or assigning outcomes. All study site staff who administer drugs, assess patients and process laboratory samples do not have access to the ran-domisation code breaker.

All participants receive a 3 day course of artemether lumefantrine according to Ugandan national treatment guidelines for uncomplicated malaria. Participants are ran-domised to receive a placebo or a dose of 0.1, 0.4 or 0.75 mg/kg primaquine in addition to the AL treatment. The dose of primaquine/placebo is given at the same time as the fifth dose of AL, in the morning of day 2. To pre-serve the accuracy of lower weight-based doses, all prima-quine doses are administered in aqueous solution and measured using a sterile syringe. The placebo is aqueous solution alone. All doses including placebo are mixed with glucose-based syrup that masks the colour and taste of primaquine. All treatments are directly observed. A snack with approximately 5 g of fat is administered prior to both AL and primaquine administration to optimise absorption of AL and minimise gastrointestinal side effects with primaquine. Participants are observed for 30 minutes; treatment is readministered in any case of vomiting within this period. Repeated vomiting (>3 times) leads to exclu-sion from the study and treatment as complicated malaria according to national guidelines.

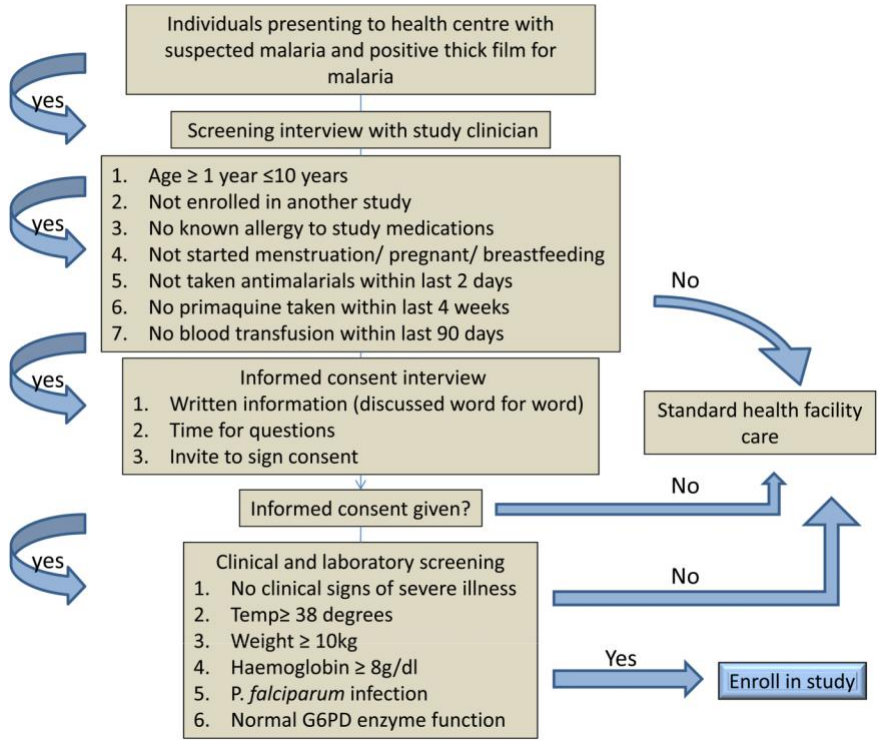

Figure 1 Enrolment and selection procedures.

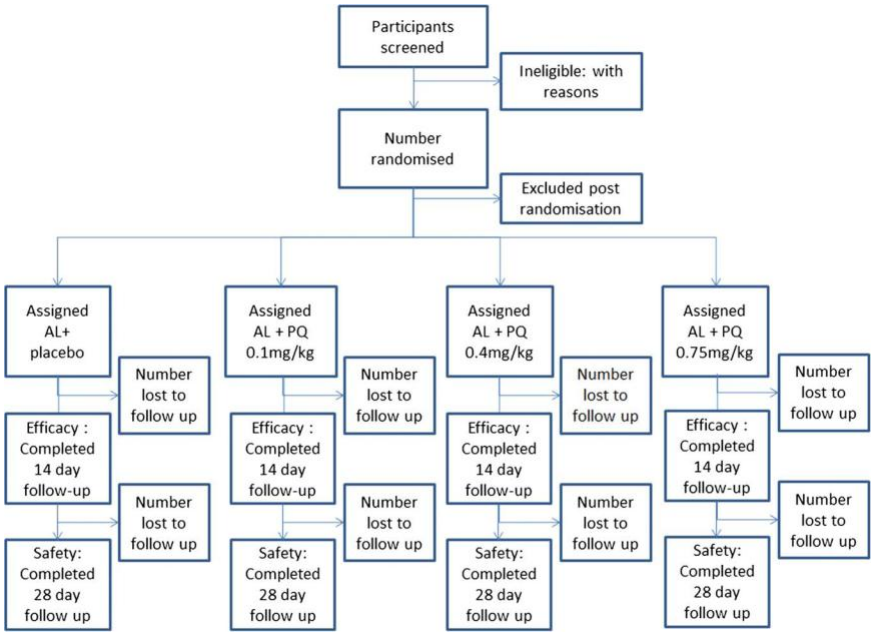

Figure 2 Participant flow diagram.

Follow-up measurements

Study participants are reviewed on days 0, 1, 2, 3, 7, 10, 14, 21 and 28 after enrolment or on any day of illness. On each of the scheduled visit days they are assessed clinically with standardised adverse event recording and blood samples are taken for microscopical detection of asexual parasites and gametocytes, molecular detection of gameto-cytes and haemoglobin measurements (table 1).

Blood smears from all visits are Giemsa-stained and 100 microscopic fields are screened for asexual parasites on days 0, 1, 2, 3, 7, 10, 14, 21 and 28. Asexual parasites are counted against 200 white blood cells (WBC) or, if fewer than 10 parasites are observed per 200 WBC, against 500 WBC. Gametocytes are recorded if observed during this screening process. On day 0, 100 microscopic fields are reread for gametocytes specifically. If gametocytes are observed, they are quantified against 500 WBC. All micros-copy readings are performed by two independent micro-scopists, if they disagree on prevalence or if density results differ by more than 25%, a third reading is requested.

Gametocytes are quantified on days 0, 2, 3, 7, 10 and 14 using quantitative real-time nucleic acid sequence-based analysis (QT-NASBA), detecting and quantifying Pfs25 mRNA. One hundred microlitres of finger prick blood is mixed with 900 µl L6 guanidine buffer (Severn Biotech, UK) and stored at −80°C until automatic nucleic acid extraction by MagNAPure (Roche) using commercial high-yield kits. The Pfs25 QT-NASBA is spe-cific for mature gametocytes with a sensitivity of 0.01–0.1 gametocytes/µl of blood when 50 µl blood samples are used for RNA extraction.[40]

Haemoglobin is measured on days 0, 1, 2, 3, 7, 10, 14, 21 and 28 using HemoCue 201+ photometers (HemoCue; Angelholm, Sweden). At each follow-up visit, study staff assess participants in an objective manner according to a clinical record form and assessment for adverse events is conducted in a prospective, systematic fashion during all visits, including the enrolment visit (eg, vomiting post-AL). All data are double-entered in real time.

Safety considerations

A protocol was developed in order to standardise the detection, investigation and management of severe haem-olysis in this trial (figures 3 and 4). A Data Safety Monitoring Board (DSMB) has been installed; clinically relevant haemolytic events, hospital admissions, blood transfusions and deaths are reported within 72 h to this DSMB.

Ethical considerations

The study protocol and informed consent forms were approved by the Makerere University School of Medicine Research Ethics Committee (protocol 2011– 210), the Uganda National Council of Science and Technology (protocol HS1056) and the London School of Hygiene and Tropical Medicine research ethics com-mittee (protocol 5987). The Ugandan National Drug Authority approved the protocol and importation of primaquine for the purposes of the study. The DSMB and Trial Advisory Committee for the study agreed to meet at predetermined stages of the study. Before the study began, local community stakeholders (including village health team and local council members) in Walukuba were consulted and a community advisory board meeting was held.

Sample size

For efficacy, the sample size calculation is based on non-inferiority of each of the two test dose arms to the comparator arm, the WHO-recommended dose of primaquine, 0.75 mg/kg. The primary outcome

Table 1 Summary of outcome measures

	Outcome measure	Description
Efficacy		
Primary	Mean number of days to gametocyte clearance (GCT)	Mean number of days per treatment arm for gametocytes to become undetectable using submicroscopic molecular testing methods (QT-NASBA). Reappearance of gametocytes after day 14 will be considered as re-infection and excluded
Secondary	Mean (±SD) area under the curve of gametocyte density per day during 14 days of follow-up	Total number of gametocytes (measured by QT-NASBA) seen over follow-up, averaged per day of follow-up (days 0–14)
	Density of gametocytes on days 7, 10 and 14	Mean number of gametocytes (measured by QT-NASBA) per treatment arm on days 7, 10 and 14
	Proportion (%) of participants with gametocytes on each day of follow-up	For each treatment arm, percentage of participants with gametocytes (measured by QT-NASBA) on each day of follow-up from days 0–14
Safety		
Primary	Mean (± SD) maximal fall (±) in Hb (haemoglobin, g/dl) from enrolment to day 28 of follow-up	Mean maximal greatest negative difference in Hb (measured by HemoCue) from enrolment value per treatment arm over 28 days follow-up
Secondary	Follow-up day of Hb nadir	Mean day of follow-up (day 0–28) per treatment arm of lowest Hb measurement (by HemoCue)
	Maximal percentage fall in Hb level compared to enrolment value	Size of maximal Hb drop (by HemoCue) during follow-up (day 0–28) from enrolment value, divided by enrolment value, *100
	Percentage of participants with Hb<5 g/dl during follow-up	Percentage (number) per treatment arm during days 0–28
	Requirement for blood transfusion	Percentage (number) of children receiving blood transfusion per treatment arm during days 0–28
	Evidence of black urine	Percentage (number) of children with documented black/ dark urine with urine dipstick positive for Hb per treatment arm during days 0–28
	Incidence of serious adverse events by sign, symptom, laboratory parameter and relationship to taking study drug	Percentage (number) per treatment arm during days 0–28
	Incidence of gastrointestinal symptoms after taking study drug	Percentage (number) per treatment arm during days 2–7

GCT, gametocyte clearance time; Hb, haemoglobin; QT-NASBA, quantitative real-time nucleic acid sequence-based analysis.

measure is number of days to gametocyte clearance. The addition of primaquine (0.75 mg/kg) to ACT in Tanzania reduced the time to gametocyte clearance from 28.6 to 6.3 days (SD 6 days).[41] Allowing for a 10% loss to follow-up, a sample size of 120/arm will provide over 80% power at the 0.05 significance level to detect non-inferiority to the standard arm with a non-inferiority margin of 2.5 days, which was considered to be a clinically relevant reduction in gametocyte clear-ance time. This sample size also allows for an analysis of superiority of the efficacy of the two test dose arms to placebo.

For safety, the sample size calculation is based on superiority of each of the two test dose arms to the com-parator arm (0.75 mg/kg). For this comparator arm, Shekalaghe et al[34] found an overall mean absolute drop in haemoglobin by day 7 after treatment of 0.6 g/dl (SD 1.5). Therefore, with 80% power and at the 0.05 signifi-cance level, a sample size of 99 would be required to detect a difference in mean maximal drop in haemoglo-bin between treatment groups of 0.6 g/dl.

Data analysis

Data will be double entered in Microsoft Access and imported into Stata V.12.0 (Statacorp Ltd, Texas, USA). All efficacy analyses will be based on gametocyte detec-tion by Pfs25 QT-NASBA. Gametocyte density on days 7, 10 and 14 will be compared with the comparator arm (0.75 mg primaquine/kg) by χ^2 test. The mean duration of gametocyte carriage and 95% CI will be estimated in each treatment arm and compared with the comparator arm using a previously validated mathematical model.[42] The area under the curve of gametocyte density over time will be calculated using the method described by Mendez et al[43] For individuals who are gametocyte posi-tive at enrolment, Kaplan-Meier survival analysis will be used to compare the decline in gametocyte prevalence.

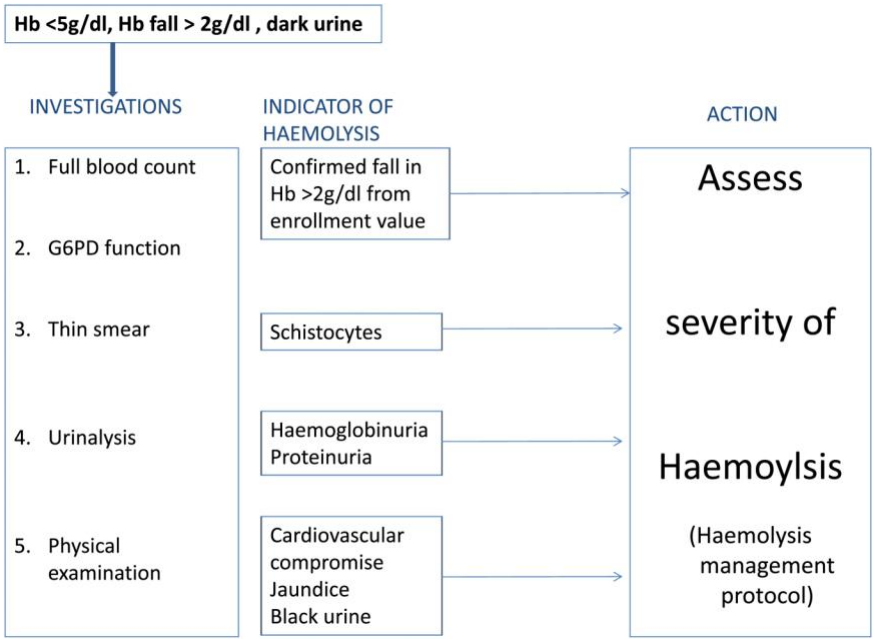

Figure 3 Procedure for investigation of suspected haemolysis.

The primary safety outcome, mean maximal fall in haemoglobin concentration during 28 days of follow-up will be assessed for each treatment arm. Pair-wise com-parisons will be made between each of the treatment arms and compared with the comparator arm using unpaired t tests.

DISCUSSION

In the 2010 edition of the Guidelines for the Treatment of Malaria, the WHO recommends that a single dose of 0.75 mg/kg primaquine is added to ACT in malaria elimination programmes and for epidemic control, provided the risks of haemolysis in G6PD-deficient patients are considered. This guidance was recently updated to recommend a lower dose of 0.25 mg/kg primaquine without G6PD testing for new malaria elimination programmes and to prevent the spread of artemisinin resistance.[31] The revision was based largely on grey literature and historical data rather than on recent clinical trials and few of the data are in the public domain.[44] There have been no formal dose-finding studies using contemporary tools and stan-dards for the measurement of drug efficacy and safety for the combination of ACTs and primaquine. In the current study, we aim to provide these urgently needed efficacy data and provide safety data for individuals with normal G6PD enzyme function.

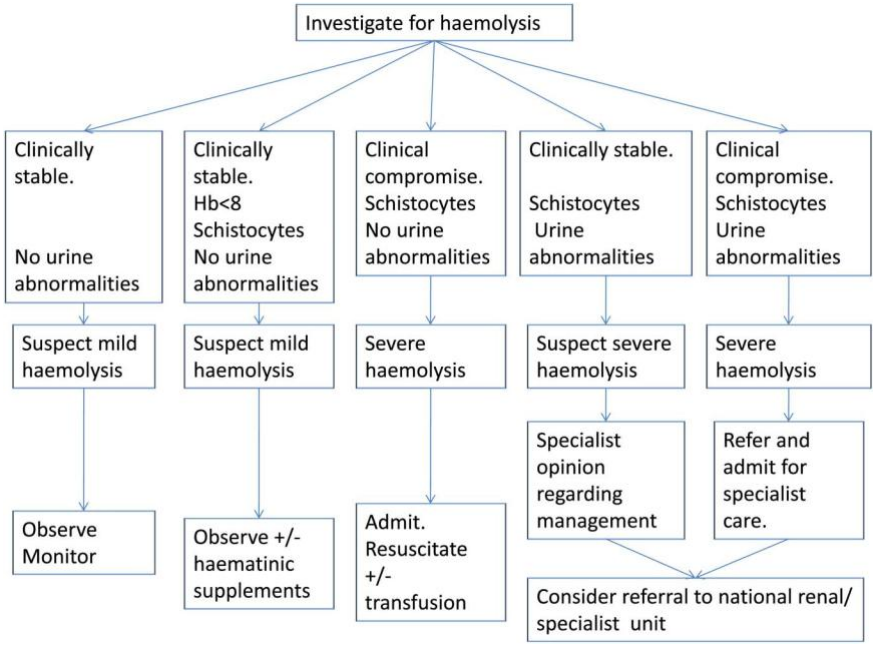

Figure 4 Procedure for management of haemolysis.

Relatively few trials have been designed specifically to test gametocytocidal drugs in vivo. Standardised proto-cols and trial designs for assessing the efficacy of drugs targeted against asexual parasites[45 46] are not suitable to assess gametocytocidal drugs, where the main outcome is transmission-blocking activity rather than clinical or parasitological cure. There is no agreement on the best tools to quantify gametocyte carriage. Many trials have used microscopy to measure gametocytes[26–28 47 48] while it has been known for decades that microscopy is notori-ously insensitive for detecting gametocytes.[49] Gametocytes typically circulate at densities that are ≤1% of asexual parasite densities.[16 50] Nevertheless, gameto-cytes are often simply recorded while screening for asexual parasites. If slides are specifically read for game-tocytes, the number of microscopic fields that is screened is mostly the same as that for asexual para-sites.[51] As a consequence, gametocytes measured micro-scopically by routine underestimate the total gametocyte prevalence by up to 10-fold.[16 17 21 22] In the current study, gametocytes are quantified with the most widely used quantitative molecular gametocyte detection method, QT-NASBA that has an estimated sensitivity of 0.01–0.1 gametocytes/µl blood in the blood sample taken.[40] The use of this sensitive molecular method will increase the power of our efficacy estimates since up to 90% of symptomatic malaria patients may harbour (sub-microscopic) gametocyte densities prior to the initiation of treatment.[16]

Gametocyte density is associated with the likelihood of mosquito infection and some of the lowest gametocyte densities may therefore be unlikely to result in mosquito infections. In general, there are limitations to which gametocyte prevalence or density can be used to predict mosquito infection rates. The fitness or infectivity of gametocytes is variable, especially after treatment.[19 52 53] Very early studies demonstrated that primaquine may render gametocytes non-infectious several days before they are cleared from the circulation.[30 54 55] The only approach to directly measure transmission-blocking potential involves assessing the infectiousness of the par-ticipant's blood to mosquitoes using the membrane feeding assay or direct skin feeding assays,[56] the latter being described by early malariologists.[57 58] However, the capacity for mosquito feeding assays is not widely available and repeated assessments of infectiousness on the same patients have never been performed as part of clinical trials. This is partly because of ethical concerns related to repeated venous bleeding in young children, and partly because of the complexity of mosquito hus-bandry when large numbers of mosquitoes are required for robust transmission estimates.[59] In the absence of biomarkers, using the prevalence and density of gameto-cytes after treatment is the most pragmatic approach to assess the transmission-blocking efficacy of drugs across a variety of malaria endemic settings.

To assess the safety of the 8-aminoquinoline drugs, there must be a clear definition of the risk of haemolysis and how it should be measured.[31 60] The safety profile may best be defined by the incidence of endpoints that could compromise health, such as signs of severe haem-olysis, and the need for interventions such as haematinic drug administration, hospitalisation or blood transfu-sion. These events, however, are rare and changes in haemoglobin concentration may be a more sensitive primary safety outcome for standard clinical trials. In a recent Cochrane review of randomised controlled trials of primaquine's efficacy, only one trial[25] was found to have measured the haemoglobin concentration to assess safety.[61] In this current study, clinically relevant safety endpoints have been selected and a standardised pro-cedure is in place for the investigation and management of severe haemolysis. A shortcoming of the current study is that safety data are most urgently needed in the most vulnerable group, G6PD-deficient individuals. For ethical reasons this group was excluded. The authors consider that the priority is first to determine the minimal effective dose in a G6PD normal population before G6PD-deficient individuals are exposed to this low dose of primaquine to assess safety.

The ultimate evidence for a beneficial role of prima-quine in reducing malaria transmission would come from trials assessing the effect of the drug on measures of community-level transmission. Once a safe and effica-cious dose of primaquine in combination with ACTs is established, the next step involves designing these com-munity trials. Treatment of symptomatic cases could play an important role in reducing the spread of (resistant) malaria strains from symptomatic patients.[62] However, because of the large pool of asymptomatic parasite car-riers in all endemic settings[63] and their importance in defining transmission potential, any effect of prima-quine on community-wide transmission will be limited if administration is restricted to symptomatic cases. Other strategies such as pro-active screening and treatment and (focal) mass drug administration may have a larger impact in some settings.[64] This trial forms the starting point for defining the optimal dose of primaquine for use in transmission-blocking interventions.

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Contributors ACE, SGS, SY, NJW, TB and CD have conceived and designed the study and participated in logistical planning together with EW and MK. EW provided the statistical support for the sample size estimates and the design of the statistical analysis. TB provided the expertise for submicroscopic gametocyte measurement. ACE organised the ethical applications, community sensitisation and study implementation and wrote the manuscript together with TB and CD. All authors have read and approved the final manuscript.

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Competing interests None.

Ethics approval Makerere University School of Medicine Research Ethics Committee (protocol 2011-210), the Uganda National Council of Science and Technology (protocol HS1056) and the London School of Hygiene and Tropical Medicine research ethics committee (protocol 5987).

Provenance and peer review Not commissioned; externally peer reviewed.

Trial status Recruitment began on 6 December 2011. The trial is going on.

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Study protocol for a randomised controlled double-blinded trial of the dose-dependent efficacy and safety of primaquine for clearance of gametocytes in children with uncomplicated falciparum malaria in Uganda

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2.2 Protocol development

The proposed trial objectives relate to gametocyte clearance to block transmission of infection to the mosquito rather than cure of the malaria-infected individual. Standard antimalarial drug efficacy trial guidelines (201) focus on clearance of asexual parasites and clinical outcomes. An innovative part of this work was to design a method for assessing transmission-blocking efficacy and safety that might be transferrable to future trials of transmission-blocking drugs. Although there were a small number of instances where transmission-blocking had been evaluated already, a Cochrane review in 2012 highlights the heterogeneity of methods that had been employed in such assessments prior to this trial (197).

Unique ethical issues are raised when trialling a drug whose action is primarily for the benefit of the community rather than the individual participant (199, 202). The individual treated with single dose primaquine benefits only indirectly from the community effect, rather than directly from the drug effect. Standard passive detection of adverse events was considered inadequate; safety outcomes were designed to reflect the specific haematological risk of the drug. Community engagement and community stakeholder partnership were integral parts of trial implementation and there was an emphasis on exploring the ethical issues around primaquine use during engagement events.

This chapter presents the process of enquiry that led to protocol development. The trial protocol can be found in Appendix A.

2.2.1 Investigating the efficacy of primaquine: what questions to ask?

2.2.1.1 *What is the optimal dose of primaquine for transmission-blocking?*

The WHO recommended dose at the time this trial was designed was 0.75mg primaquine base/kg to a maximum of 45mg in adults (86).

The earliest studies on 8-aminoquinolines observed that single doses cleared gametocytes within a few days (77, 203). The doses used were the same as the daily causal prophylactic dose and the gametocytocidal effect was an incidental advantage. Most studies, therefore are based on this standard dose. Very few studies have assessed the efficacy of different doses to the standard 0.75mg/kg.

Gunders (in 1961) (204) used a dose of 1-2mg/kg in a cohort of 22, largely children, in Liberia and reported few adverse effects. Bunnag (in 1980) (205) compared the effect of 15mg daily for 5 days, 30mg single dose and 45mg single dose in Thai adults and found no significant difference in gametocyte clearance between doses. Pukrittayakamee (in 2004) (206) compared 0.25mg/kg and 0.5mg/kg primaquine in adults and found both to have shorter gametocyte clearance times (GCT) than artesunate containing regimens with no significant difference between the two doses.

This suggests that low doses may be as effective as higher doses. A very low dose of pamaquine (Plasmoquine), 0.02mg/kg was reported as gametocytocidal (207). This translates to a molar equivalent of 0.0164mg/kg of primaquine base.

On this basis, it is possible that the WHO dose is excessive for gametocytocidal efficacy and the lowest dose for efficacy and safety ought to be established.

2.2.1.2 *Is a standard superiority design optimal for the evaluation of lower-than-standard doses?*

Rather than the superiority design of antimalarial drug efficacy trials (201), for dose-finding, the emphasis is on assessing the efficacy of lower doses than the recommended dose. An analysis based on non-inferiority to the existing 0.75mg/kg WHO-recommended dose may be a more appropriate approach (208). This requires a larger number of participants than a dose-escalation study (209).

Possible outcomes of a non-inferiority trial arm are “non-inferior”, “not non-inferior”, or “inferior” to the comparator arm (210) (Table 2-1). Table X presents some possible inferences drawn from these outcomes with relation to the fictional trial arms. There may be difficulty interpreting the inference if a study arm has “not non-inferior” but not “inferior” outcome (210). For this reason, the non-inferiority margin was constructed with some thought to its biological relevance. Guidelines were consulted on the procedures for choice of the non-inferiority margin (211).

Table 2-1 Possible inferences from non-inferiority efficacy analysis outcomes from a fictional dose-finding study

Reference arm	Comparator arm	Non-inferiority analysis outcome	Inference
A	B	"Non-inferior"	Dose B dose is not less efficacious than dose A
A	C	"Not non-inferior"	The efficacy of dose C is not inferior to dose A but the study cannot determine whether it is equivalent to dose A
A	D	"Inferior"	Dose D is less efficacious than dose A

The non-inferiority margin represented the maximum additional number of days for which we speculated an individual might remain gametocyte positive, such that the primaquine dose administered would still be considered as efficacious as a comparator dose. Available data suggested that submicroscopically detected gametocytes (the study outcome measure) were cleared most rapidly within 11 days after 0.75mg/kg primaquine administration, although a small number of individuals still carried gametocytes beyond day 28 (63, 212). A margin of 2.5 days was selected to distinguish between doses, so, for example, clearance within 12.5 days would be seen as non-inferior to clearance within 10 days. Figure 3-1 (in Chapter 3, Results) shows graphically how trial results were interpreted using non-inferiority analysis.

2.2.1.3 *What is the optimal timing of primaquine dosing with concomitant schizontocidal treatment?*

If primaquine is to be given in clinical case treatment or mass treatment initiatives, it is logistically simpler, much cheaper and more reliable to give it at the same day as the partner asexual treatment. However, often it is given after the start of treatment to avoid exacerbating the nadir in haemoglobin associated with clinical malaria. Based on a gametocyte half-life of 4-6 days, some authors suggest giving primaquine on day 7 or 8 to capture maturing gametocytes which develop in the first few days of treatment (213). Few studies have examined the efficacy associated with the timing of primaquine treatment. Lederman (214) found a shorter GCT when primaquine was given on day 2 rather than day 0, but this was not significant. The range of regimens of primaquine administration in the literature are illustrated in Table 2-2.

Table 2-2 The range of timings of primaquine treatment in published studies

Day of primaquine administration (after asexual treatment on day 0)	Country	Comment	Author, year
0-6 (7 days)	Thailand	No detailed safety data. No adverse events reported	Pukrittayakamee, 2004 (206)
3	India	No significant adverse haematological or other events	Gogtay, 2006 (215)
0 vs 2	Indonesia	Day 2 group had faster GCT, but difference was not significant. Not powered to detect difference. No safety reporting	Lederman, 2006 (214)
2	Tanzania	Haemoglobin nadir on day 7. Worse in G6PD deficient. No symptomatic anaemia	Shekalaghe, 2007 (63)
2	Sudan	Asymptomatic cases (mass drug administration). No difference in packed cell volume on day 7.	El-Sayed, 2007 (216)
0	Colombia	No safety reporting	Alvarez, 2010 (217)
0	Myanmar	Haemoglobin *increase* by day 63 was reduced by 0.295g/dL in PRIMAQUINE-treated group. No black water or severe anaemia	Smithuis, 2010 (218)

2.2.1.4 *What pharmacokinetic properties of primaquine influence study design?*

Primaquine is extensively metabolized; less than 2% of the parent compound is excreted in the urine within 24hrs of dosing (219). Several metabolites have been identified, but it is unclear which are responsible for the action against hypnozoites and gametocytes and which for the toxic effects. The mechanism of action of primaquine remains unclear. Carboxyprimaquine is the main metabolite (104) and its formation is cytochrome CYP450-dependent (220). The 5-hydroxylated metabolite has been linked to both therapeutic efficacy and toxicity (221). New evidence suggests that that a range of hydroxylated metabolites are responsible for primaquine's efficacy in clearing liver stage and sexual stage parasites, and that this action is dependent on human liver microsome activity (222). Hydrogen peroxide (H_2O_2) generated from primaquine metabolism is hypothesized to cause parasite killing through oxidative stress. Hence, the very mechanism for primaquine's efficacy may be linked to the drug's toxicity to humans, which is also induced by oxidative stress. Other metabolites have been identified, but their function remains undetermined (89).

A prerequisite of reliable pharmacokinetic data is a robust assay for drug detection. A high performance liquid chromatography (HPLC) method devised in 1984 (104) to detect primaquine with a sensitivity of 1ng/ml has been updated by Cuong (88). Primaquine exhibits extensive tissue distribution (102, 223). Peak plasma concentration is within 1-4 hours (133, 219, 223) and the terminal half-life is 4-6 hours (133, 219).

Table 2-3 categorises studies that provide pharmacokinetic data which could affect the design of a primaquine efficacy and safety trial. The ethnicity, age, sex and symptomatology of participants may influence outcomes as well as the methodology of the study, such as dosing schedule, combination drugs used and method of administration.

Table 2-3 Summary of pharmacokinetic data that affect study design

Variable	Numbers studied	Detail	Reference
Ethnicity	5-11	Thais +/- G6PD, Caucasians. Basic PK no significant difference 45mg stat	Fletcher 1981 (219)
	18	Australians in this study had much higher clearance (lower AUC and C^{max}) compared to Thais from Singhasivanon 1991, even considering weight difference	Elmes 2006 (223)
	20	Vietnamese (30mg) values similar to Mihaly (45mg) in having substantially lower C_{max} and AUC than Thai study (15mg)	Cuong 2006 (88)
Age & sex	7-9	Adult males	Edwards 1993 (133)
	5	Adult males	Mihaly 1984 (104)
	5	Adult males	Mihaly 1985 (102)
	6-30	Adult males	Fletcher 1981 (219)
		Thai Females higher AUC and C_{max} than males (15mg dose)	Singhasivanon 1991 (224)
	18	9 male, 9 female (Australian) healthy, single dose (30mg) weight-adjusted results: no difference in AUC, C_{max}, CL/f or $t_{1/2}$	Elmes 2006 (223)
	20	No significant difference between 10 men and 10 women (geographic mean ratio of C_{max} 0.89 and AUC 0.80, both non-significant). No change in dose required.	Cuong 2006 (88)
Drug interaction	30	Chloroquine: increases production of methaemogobin (time scale compatible with primaquine metabolite)	Fletcher 1981 (219), Cowan 1964 (225)
	9	Mefloquine: no significant effect	Edwards 1993 (133)
	7	Quinine: reduction of carboxy-metabolite AUC	Edwards 1993 (133)
		Artesunate:	
	20	Grapefruit juice increased mean C_{max} (23%) and AUC (19%). Highly	Cuong 2006 (88)

		variable between individuals—avoid co-administration	
Malaria interaction	9	Clinical malaria reduced oral clearance of primaquine, and reduced t_{max}	Edwards 1993 (133)
Repeated dosing	5	Accumulation of carboxy-primaquine at 14 days with higher C_{max} and AUC.	Ward 1985(226)
Administration with food	5-9	All starved overnight	Mihaly 1984, 1985 (102, 104), Ward 1985 (226), Edwards 1993 (133)
	18	All given with food min 30% fat	Elmes 2006 (223)
	20	Food (bread and butter-28g fat 5 mins before dose) increased the C_{max} by 26% and the AUC by 14%. All also given 300ml water). All given a meal 4 hours *after* dose. Comment that increased bioavailability with food is too modest to worsen adverse events also reduces GI side effects and could be useful given resistance	Cuong 2006 (88)
Dose assessed in study		15mg, 30mg, 45mg	Mihaly 1985 (102)
		45mg for carboxyprimaquine data	Mihaly 1984 (104)
		15mg Thai male female difference and higher C_{max} and AUC than other studies	Singhasivanon 1991 (224)
		30mg Australian male vs female	Elmes 2006 (223)
		30mg Vietnamese male, female food, grapefruit juice	Cuong 2006 (88)

Abbreviations: PK = pharmacokinetic; AUC = area under the plasma concentration-time curve; C_{max} = the maximum concentration of a drug in the blood after the drug has been administered and before the administration of a second dose, i.e., the maximum peak plasma concentration; CL/f = total clearance of a drug from the plasma after oral administration, i.e., the mean oral clearance; $t_{1/2}$ = elimination half life

We can conclude that from existing data that the same dose can be given in males and females and it should be given with food. Data is lacking on the effect of artesunate

derivatives on primaquine pharmacokinetics and most data available is in adults. More data is required specifically in African children.

Pharmacokinetic studies require frequent blood sampling and particularly in children, this must stand up to ethical scrutiny. Using population pharmacokinetic models, an optimal sampling schedule can be defined to maximize the information gained from the smallest possible number of blood samples (227).

2.2.1.5 *What is the ideal transmission setting for trials of primaquine for transmission-blocking?*

A main determinant of drug efficacy in clearing gametocytes is the pre-treatment gametocytaemia (228) and this varies with age (229) and the entomological inoculation rate (EIR) (230). Therefore, the effect of primaquine in interrupting transmission may vary between transmission settings. Given the heterogeneity of transmission intensity over time and place and the complexity of the determinants of transmission efficiency, data on primaquine's gametocytocidal efficacy in a range of settings will be of value to inform further modelling and eventually to inform policy decisions. We do not have convincing epidemiological data on the effect of primaquine in reducing malaria transmission at community level. Would primaquine have any significant effect in a high transmission setting where asexual parasite rates are high and fuel ongoing gametocyte production? Clyde's work in the 1960s demonstrated high impact of a primaquine-including regimen on transmission reduction in a high endemic region of Tanzania (83) . However, the relative contribution of primaquine cannot be ascertained due to lack of a control arm (Section 1.2.1.3.1).

Table 2-4 summarizes the transmission settings for the range of primaquine trials at the start of this thesis.

Table 2-4 The transmission setting for trials of primaquine as a gametocytocidal agent

Transmission setting (EIR* if available from reference)	Country	Author, year
Low	Thailand	Bunnag, 1980 (205) Chomcharn, 1980 (231)
Low	Indonesia	Kaneko, 1989 (232)
Moderate	India	Gogtay, 1999 (233)
Low	Thailand	Suputtamongkol, 2003 (234)
Low	Thailand	Pukrittayakamee, 2004 (206)
Moderate	India	Gogtay, 2006 (215)
Low-moderate	Indonesia	Lederman, 2006(214)
High (91)	Tanzania	Shekalaghe, 2007 (63)
High, seasonal	Sudan	El-Sayed, 2007 (216)
Low	Colombia	Alvarez, 2010 (217)
Low-moderate	Myanmar	Smithuis, 2010 (218)

**EIR = entomological inoculation rate (the number of infective mosquito bites per person, per year)*

Definition of transmission settings: Low transmission, Plasmodium falciparum parasite rate (PfPR) 1-10%; moderate transmission, PfPR 10-35%; high transmission, PfPR ≥ 35%; seasonal transmission, malaria transmission occurs only during some months of the year (235, 236)

The majority of these studies have been conducted outside sub-Saharan Africa. At the time of thesis design, no African countries had been defined as in pre-elimination of elimination phase. African data is now clearly relevant, as malaria elimination is now firmly on the agenda for an increasing number of states (1).

For dose-finding studies, high transmission settings may be advantageous. Potentially, the higher gametocytaemias encountered would provide more data, and faster recruitment, for a comparison of efficacy between different doses of primaquine. This difference may not be as readily discernible in low-transmission settings. Translation into policy needs consideration. There may be isolated moderate transmission settings where primaquine will be used (such as on islands, or for outbreak control (194) , but most recommendations have been for primaquine use in elimination or pre-elimination settings of low malaria transmission (Section 1.2.1.4.1).

2.2.1.6 *Is the schizontocidal drug combination important?*

Schizontocidal drug failure results in prolonged clearance time or recrudescence of the asexual parasitaemia. Hence, the effect of a gametocytocidal drug is offset by newly forming gametocytes. Interpretations of the efficacy of primaquine from studies have been conducted with a failing regimen against asexual parasites (215, 234) should be drawn with caution.

Drugs with short duration of action can result in higher incidence of re-infections, and a new source of gametocytes compared with drugs with prolonged effect against asexual parasites. Hence, artemisinin combinations containing lumefantrine or piperaquine may show lower total gametocyte prevalence during follow up than shorter acting combinations.

For efficacy trials, highly effective asexual stage treatment should be used and the half-life of the schizontocidal drug should be considered when assessing the gametocyte prevalence during follow up.

2.2.1.7 *What is the ideal outcome measure for efficacy?*

Gametocytocidal efficacy can be measured as the prevalence or density of gametocytes during follow up by microscopy. Gametocyte density can vary considerably from day to day as seen in experimental infections (81, 237) . Therefore, point density and prevalence comparisons are less informative than cumulative measurements.

Mendez (238) defined the log10 of the mean area under the curve of gametocyte density over time per day as an indicator of the total infectious potential of an individual treatment group. This has been used subsequently in primaquine trials (63).

Gametocyte density can be measured using molecular detection methods such as QT-NASBA, a real time quantitative nucleic acid sequence based amplification detects gametocytes at densities down to 20-100 per ml (237). This is a factor of 10 below the theoretical limit for infectiousness to mosquitoes, given assumptions around the relationship between gametocytaemia and the mosquito blood meal. This higher level of detection is useful to highlight significant differences between treatment arms as shown in Figure 2-1.

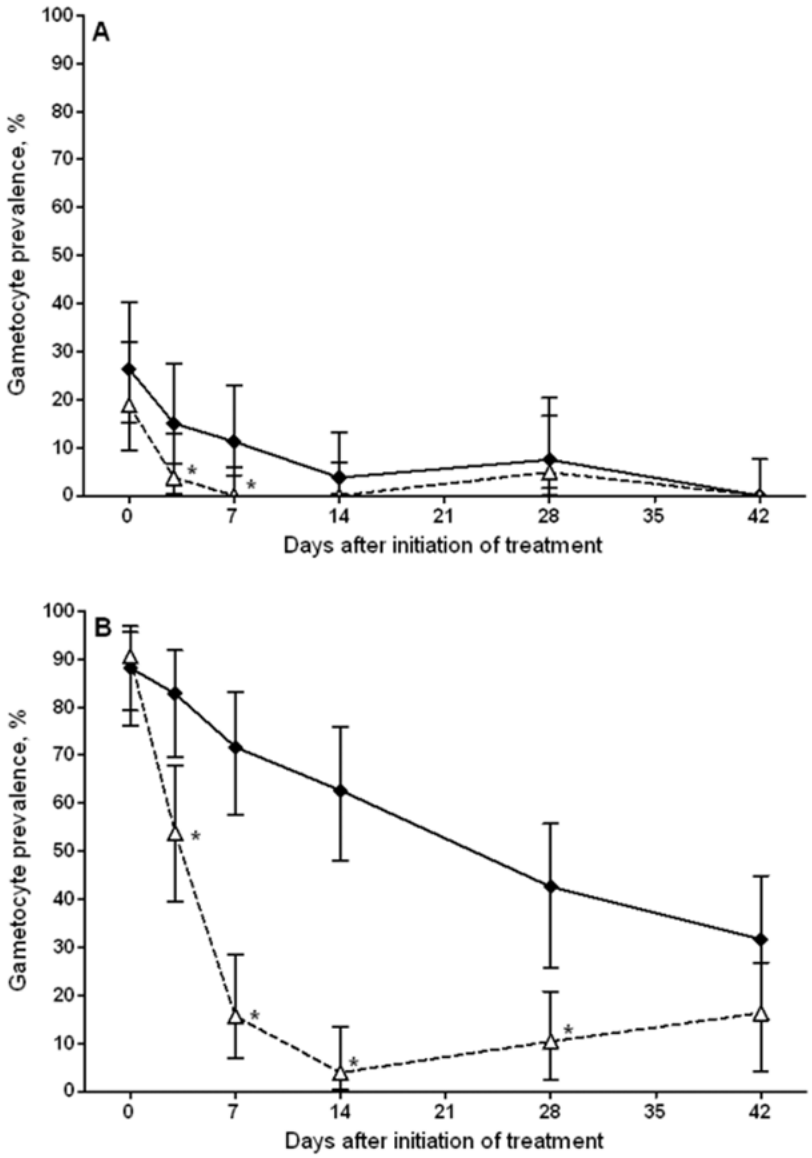

Figure 2-1 Differences between treatment arms with microscopic and submicroscopic detection methods, from Shekalaghe *et al*, PLosOne, 2007 (63)

Gametocyte prevalence by microscopy (A) and Pfs 25 QT-NACBA (B). *Gametocyte prevalence for SP+AS (closed diamonds, solid line) and SP+AS+PQ (open triangles, broken lines) treated children. Bars indicate the 95% confidence intervals around the proportions. *indicates a statistically significant difference between the two treatment arms. (From original manuscript (63))*

To measure the true transmission potential following primaquine treatment, ideally, transmission experiments should be performed, with sporogony as an outcome, namely

oocyst numbers in the mosquito midgut or presence of sporozoites in the mosquito mouth parts. Table 2-5 illustrates the outcome measures used in published studies of primaquine as a transmission-blocking agent. The measures represent how primaquine impact can be measured on gametocytes and through the process of sporogony to sporozoite production and community parasite rate.

Table 2-5 Outcome measures for primaquine as a transmission-blocking agent

Outcome measure	Numbers studied	Author, year
Microscopic gametocytes only	315	Bunnag, 1980 (205)
	176	Pukrittayakamee, 2004 (206)
	90	Gogtay, 2006 (215)
	117	Lederman, 2006 (214)
	468	Alvarez, 2010 (217)
	808	Smithuis, 2010 (218)
Submicroscopic gametocytaemia	104	El-Sayed, 2007 (216)
	108	Shekalaghe, 2007 (63)
Oocysts in mosquito midgut	10	Jeffery, 1956 (82)
	10	Gunders, 1961 (204)
	n/a (in vitro)	Chotivanich 2006 (239)
Sporozoite prevalence	12	Burgess, 1961 (77)
	3	Rieckmann, 1968 (78)
Community parasite rate	>15 000	Clyde, 1962 (83)

Clearly, to demonstrate the consequence of gametocytocidal therapy on infectiousness to mosquitoes, a measurement of gametocyte density or prevalence is only a proxy measure. Furthermore, simply quantifying gametocytes after treatment might not provide a full representation of drug effect. Detection by microscopy does not distinguish between gametocytes that may be viable and infectious, or that may be sterilized or dead due to the drug effect. Where sporogony has been measured, in previous studies, gametocytes are still detectable long after sporogony is inhibited (within 3 days of primaquine) (77, 78). This suggests that gametocyte density/prevalence might be a conservative estimate of transmission potential following primaquine treatment. As long as this is considered, since it is a logistically simple measurement to conduct, the use of gametocyte quantification might be justifiable as an outcome measure in primaquine efficacy trials.

2.2.1.8 *What should be the defined duration of follow-up?*

Early studies predating the onset of widespread anti-malarial drug resistance show complete gametocyte clearance within 3-14 days of primaquine treatment (77, 78, 204) and total inhibition of sporogony within 3 days of primaquine (77, 204). In subsequent years, microscopic gametocyte clearance has been noted by 14-28 days with regimens varying in efficacy against asexual parasites (205, 206, 217, 218). Submicroscopic gametocyte clearance with primaquine is detectable in 3.9% to 6.4% on day 14 (206, 216). Following that, there is a detectable increase which may be due to re-infection or recrudescence of asexual parasitaemia (Figure 6). 28 days might, therefore, be a reasonable duration of follow up for a dose-finding study.

2.2.1.9 *In what clinical context should primaquine efficacy be assessed?*

The optimal clinical setting for the implementation of primaquine-based intervention is undetermined (199) . For malaria elimination/eradication, primaquine may be used in mass treatment of asymptomatic individuals in the community or in case-based treatment. Is it

ethical, then, to test its efficacy only in trials of clinical cases? Are results from trials on patients with uncomplicated clinical malaria translatable to effects on asymptomatic parasitaemic individuals? Clinical (symptomatic) malaria itself may have an effect on the pharmacokinetics of primaquine (133) and on gametocyte immunity (230) impacting the magnitude and rate of post-treatment gametocytaemia reduction compared to in asymptomatic infection. Symptomatic malaria also impacts the haemoglobin so extrapolation of safety data from trials in symptomatic cases should be done with caution. Notably, WHO guidelines for primaquine as a gametocytocide are for the treatment of clinical cases (Section 1.2.1.4.1). This is how primaquine has been used in South American countries for decades, where the endemicity of falciparum malaria has been reducing progressively (1).

Given that the prevalence of gametocytaemia in asymptomatic infection is low, although highly variable (37), initial dose-finding studies in the context of clinical symptomatic malaria will have more signal to compare different doses, i.e., there will be more individuals with measurable endpoints. Primaquine use in symptomatic cases is a consideration in epidemics. Furthermore, especially if primaquine is to be used in mass treatment initiatives, it is important that safety analysis is available in the context of controlled trial settings.

2.2.1.10 *What important confounding factors should be controlled for in a trial of primaquine efficacy?*

Individuals with G6PD deficiency are at risk of haemolysis with primaquine. Given that G6PD gene polymorphisms are conserved in malaria endemic regions (96, 162), and it is considered that screening for G6PD deficiency for gametocytocidal primaquine treatment may not be necessary or practical, it is important that information on safety in G6PD deficiency is available. Therefore, this should not be controlled for, but can be part of a sub-analysis. G6PD deficiency inheritance is sex-linked, so ideally, gender should be stratified for in enrolment. Of

note G6PD deficiency is not the only predisposing condition for haemolysis with primaquine (63).

Pre-treatment gametocytaemia increases the probability of prevalence of gametocytes following treatment (228). This should be stratified for in analysis.

Age may affect baseline gametocytaemia and anti-gametocyte immunity (230) so age-stratification is important in both enrolment and analysis. A cut off of age 5 may be reasonable because in a similar transmission setting, 5-7 was the upper age group where immune responses appear to impact infection (240).

2.2.2 Investigating the safety of primaquine as a gametocytocidal agent. What questions to ask?

2.2.2.1 *What are the ethical issues when giving a treatment which does not contribute directly to the health of the individual?*

Primaquine use has been advised by the WHO for transmission-blocking. This is an off-label use of the drug. This recommendation does not constitute evidence that it is ethical to give a medication to individuals for the sake of reducing malaria transmission in the community. Since those individuals do not benefit directly from the treatment, the ethical concern is related to whether there is any risk to those individuals. There is, however, a lack of data to inform at what dose and in which settings primaquine could be used with safety and efficacy. Therefore, this clinical equipoise is the proposed justification for conducting research on this intervention. It is crucial that proposed trials undergo ethical review that considers this lack of direct benefit to the individual, both in their clinical settings and sponsor institutions.

2.2.2.2 *What is an acceptable level of haemolysis post primaquine treatment?*

Arguably, no degree of haemolysis should be accepted following a treatment that is not of benefit to the individual. The haemolysis following primaquine is transient, as shown by

Shekalaghe *et al* (2007, Figure 3) (63) . Data are lacking to define the expected size of haemoglobin nadir post treatment, whether this differs with a range of starting haemoglobin values and crucially, the risk of adverse outcomes from primaquine-associated haemolysis. In Tanzania, the nadir in haemoglobin following primaquine treatment (0.75mg/kg primaquine base) was on day 7, when it was 5.2% lower than on enrolment (63).

Review of the current literature indicated that none of the trials using single dose primaquine 45mg reported any severe adverse events associated with primaquine use, including no transfusion requirement and no documentations of black urine secondary to haemolysis. There was, however, significant heterogeneity in the safety metrics that were collected. Few studies measured haemoglobin concentration or haemolysis specifically; non-specific adverse event reporting was common. There was one case of severe anaemia (Hb <5g/dL) without clinical compromise in a Tanzanian study (101) where the mean fall in haemoglobin following treatment was 2.5g/dL.

Data regarding the primaquine dose-related reduction in haemoglobin are required if its use may become more widespread in populations with G6PD deficiency.

2.2.2.3 *How will G6PD deficient individuals be identified?*

2.2.2.3.1 Fluorescent spot test

An inclusion criterion for trial participation was normal G6PD enzyme activity. This was defined as normal fluorescence with the fluorescent spot test (241). If fluorescence was reduced, this was interpreted as G6PD deficiency and the participant was excluded. The assay kit used (N Dimopoulos SA, Greece, formerly R & D diagnostics) shows fluorescence if G6PD enzyme activity is greater than approximately 20% of normal reference enzyme activity (Figure 4-1, Section 4.3.1.1). This cut off is particularly low, with reference to the WHO classification of severity (Table 1-1), and falls in the range of moderate to mild deficiency.

2.2.2.3.2 G6PD genotype

DNA was extracted from blood spots on Whatman filter papers, then amplified using the polymerase chain reaction and detected using primers that characterise the G6PD A- allele (202G-> A and 376A->G). Figure 4-2 (Section 4.3.1.2) shows the amplified products run on an electrophoresis gel after digestion with restriction enzymes. 376A->G characterises the change from G6PD B (wild type) to A, and 202G-> A characterises the change to A- variant. The presence of both mutations is required for designation as A- variant.

2.2.2.4 *What safety outcomes are important?*

Haemolysis can be measured by the fall in haemoglobin and the presence of haematological indicators, such as reticulocytosis, haematocrit and lactose dehydrogenase levels. Intravascular haemolysis may be evident on a blood film, exhibiting schistocytes.

The outcomes of haemolysis can range from acute or chronic asymptomatic anaemia, symptomatic anaemia requiring transfusion, transient black urine (haemoglobinuria), or black urine with renal failure.

For operational purposes, it is most useful to measure the level of the haemoglobin along with any evidence of adverse outcome of haemolysis, rather than haematological indicators which could be affected by factors other than the drug treatment itself.

Monitoring and reporting of any adverse events during the trial, which may or may not be related to drug treatment should form an integral part of the trial.

2.2.2.5 *Safety in pregnancy and breastfeeding*

Primaquine is contra-indicated in pregnancy because of the risk of haemolysis leading to adverse outcomes for both the mother and the unborn child (74). Given that it is secreted into breast milk, it is contra-indicated in breast feeding despite a lack of data (242). The

implications for widespread community interventions are that a significant proportion of female community members might be excluded from treatment.

2.2.2.6 *What is the optimal sampling framework for both safety and efficacy?*

The frequency of blood sampling was designed to capture the expected day of the nadir in both haemoglobin and gametocyte prevalence/density. Sampling on day 2 was important as a baseline, being the day primaquine/ placebo was administered and it appeared that a nadir in haemoglobin might be expected around day 7 (63). In a Tanzanian population, gametocyte prevalence declined until day 14, after which, an increased prevalence was attributed to re-infections (63). An additional data point on day 10 was proposed, to capture changes prior to day 14, then weekly samples were taken until day 28. If children were still anaemic on day 28, it was proposed that they would have further weekly sampling until resolution.

2.2.2.7 *What additional trial eligibility criteria need consideration?*

At the time of trial design, the lower age limit for primaquine use on the drug label was 1 year and this was reflected in WHO guidelines (243), so this was the lowest age for trial recruitment. To exclude the risk of administering primaquine in pregnancy, discussion was held with Ugandan public health clinicians and community representatives and 10 years was the selected upper age limit. The cut-off haemoglobin level for enrolment was 8g/dL, to exclude children with severe anaemia and significant haematological co-morbidity, and to minimise risk from potential primaquine-induced haemolysis.

2.2.3 Conclusions that can be drawn to inform study design

In summary, the following conclusions were reached regarding study design:

Table 2-6 Conclusions from evidence-based study design

Trial Criterion	Conclusion
Trial design	Parallel-arm randomized controlled trial with double blinding
Analysis approach	Non-inferiority Stratified by age and sex and gametocyte prevalence at enrolment
Primaquine reference and comparator dose	0.75mg/kg versus lower doses: 0.4mg/kg, 0.1mg/kg, and placebo
Timing of primaquine dose	Day 2
Schizontocidal drug combination	ACT recommended by local Ministry of Health (artemether-lumefantrine)
Efficacy outcome measure	Gametocyte prevalence over time, gametocyte clearance time
Safety outcome measure	Primary: mean fall in haemoglobin after treatment Secondary: requirement for transfusion, haemoglobin <5, presence of black urine
Duration of follow up	14 days for efficacy, 28 days for safety
Transmission setting	Moderate (EIR* 1-10)
Clinical context	Symptomatic uncomplicated *Plasmodium falciparum* malaria

**EIR = entomological inoculation rate (number of infective mosquito bites per person, per year)*

2.3 Development of a Primaquine working group

Following the development of this thesis, a clear gap in the malaria elimination research agenda was identified. A working group was established to focus on the research agenda and operational priorities for the use of single low-dose primaquine as a transmission-blocking intervention (described in Section 3.3.4.1). The group's remit was defined principally with questions around primaquine use in Africa, but outputs became transferable to other regions where *Plasmodium falciparum* elimination is being targeted.

2.3.1 RESEARCH PAPER 2: Publication of the rationale for using primaquine to interrupt malaria transmission in Africa

The report from the first primaquine working group meeting was peer reviewed and published in the Malaria Journal(199).

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SECTION A – Student Details

Student ID Number	257918/RITD	**Title**	Dr
First Name(s)	Alice Chijioke		
Surname/Family Name	Eziefula		
Thesis Title	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Malaria Journal		
When was the work published?	30th October 2012		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	**No**	Was the work subject to academic peer review?	**Yes**

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Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	ACE, RG and CD wrote and edited the manuscript, together with RG and CD. I acted as a meeting rapporteur along with MH and JH were meeting rapporteurs. All authors read and approved the final manuscript.

SECTION E

Student Signature	Chi Eziefula 
Date	18th September 2019

Supervisor Signature	
Date	23rd September 2019

MALARIA JOURNAL

MEETINGREPORT Open Access

Rationale for short course primaquine in Africa to interrupt malaria transmission

Alice C Eziefula[1], Roly Gosling[2], Jimee Hwang[2,3], Michelle S Hsiang[2,4], Teun Bousema[1], Lorenz von Seidlein[5] and Chris Drakeley[1*] on behalf of the Primaquine in Africa Discussion Group

Abstract

Following the recent successes of malaria control in sub-Saharan Africa, the gametocytocidal drug primaquine needs evaluation as a tool to further reduce the transmission of Plasmodium falciparum malaria. The drug has scarcely been used in Africa because of concerns about its safety in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency. The evidence base for the use of primaquine as a transmission blocker is limited by a lack of comparable clinical and parasitological endpoints between trials. In March 2012, a group of experts met in London to discuss the existing evidence on the ability of primaquine to block malaria transmission, to define the roadblocks to the use of primaquine in Africa and to develop a roadmap to enable its rapid, safe and effective deployment. The output of this meeting is a strategic plan to optimize trial design to reach desired goals efficiently. The roadmap includes suggestions for a series of phase 1, 2, 3 and 4 studies to address specific hurdles to primaquine's deployment. These include ex-vivo studies on efficacy, primaquine pharmacokinetics and pharmacodynamics and dose escalation studies for safety in high-risk groups. Phase 3 community trials are proposed, along with Phase 4 studies to evaluate safety, particularly in pregnancy, through pharmacovigilance in areas where primaquine is already deployed. In parallel, efforts need to be made to address issues in drug supply and regulation, to map G6PD deficiency and to support the evaluation of alternative gametocytocidal compounds.

Keywords: Plasmodium falciparum, Malaria, Primaquine, 8-aminoquinoline, Transmission, Gametocyte, Glucose-6-phosphate dehydrogenase deficiency, G6PD, Africa

Background

Current World Health Organization (WHO) guidelines recommend the "addition of a single dose of primaquine (PQ) (0.75 mg/kg) to artemisinin-based combination therapy (ACT) for uncomplicated falciparum malaria as an anti-gametocyte medicine, particularly as a component of a pre-elimination or an elimination programme" [1]. However, unlike recommendations for other anti-malarial treatments this does not come with the supporting statement "Strong recommendation, high quality evidence". This is because there are limited data to suggest that primaquine is safe and efficacious for this use, especially to support regulation and licensure. This is striking given that primaquine has been in the anti-malarial drug arsenal since the 1950s and historical studies strongly suggest that primaquine is highly effective at blocking transmission. Worldwide, 20 countries include primaquine as first-line treatment for Plasmodium falciparum in their national policy. None of these countries are in Africa [2].

There are an increasing number of reports of declining transmission intensity in many parts of sub-Saharan Africa, bringing malaria transmission to pre-elimination levels in some countries. There is also increasing recog-nition that additional strategies aimed specifically at the transmission stages of P. falciparum are required both to further reduce transmission and to sustain the gains made by current control efforts. The previously high levels of malaria transmission may be one of the main reasons why primaquine has not been used widely in Africa, with only very frequent delivery of the drug being likely to have any impact on transmission [3]. However, the most likely reasons for the limited use of primaquine in Africa are concerns over safety, given the conservation

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of the glucose-6-phosphate dehydrogenase (G6PD) deficiency polymorphism in the population.

Using an anti-malarial drug with the goal of inter-rupting malaria transmission rather than clinical cure necessitates a clearly-defined assessment of safety and efficacy with benefits at the individual level and at the community level being considered. For primaquine, the optimal dose to achieve such endpoints remains undeter-mined. The recommended 0.75 mg/kg dose is associated with significant haemolysis in some susceptible indivi-duals [4-6], but this dose may well be excessive for the transmission-blocking activity [7]. For the purpose of comparison, doses in this report that are expressed as a milligram per kilogram (mg/kg) equivalent assume an average adult weight of 60 kilograms.

The limited safety data available on single dose primaquine has lead to the requirement of prior testing for G6PD deficiency and pregnancy to avert risk. The necessity for this additional testing has a significant impact on the feasibility, cost effectiveness and the achiev-able population coverage of large scale primaquine-based interventions. More information on the consequences of single-dose primaquine administration on individuals/ populations with a relevant range of G6PD enzyme ac-tivity levels is required urgently if 8-aminoquinolines are to be deployed to interrupt transmission.

Meeting objectives

With these issues in mind, a meeting of experts was convened to review and discuss existing data on the use of primaquine in Africa for transmission-blocking and to examine the road-blocks that could be overcome to enable and inform its safe use.

Specific objectives of the meeting were to:

1. Identify key road-blocks to deployment of short course primaquine or similar drugs in Africa to reduce transmission of falciparum malaria.
2. Reach consensus on study endpoints so as to maximize comparability between transmission prevention studies.
3. Generate a list of deliverables that will move forward deployment of primaquine in Africa.

Meeting sessions

Country program perspectives and potential use for primaquine

Chris Drakeley and Roly Gosling introduced the meeting by providing the current context for the use of prima-quine and highlighting the fact that the reductions in malaria transmission that have been described in many sub-Saharan African settings may well be linked to in-creasing spatial, temporal and even demographic hetero-geneity in infections. Spatial targeting of control efforts is likely to make interventions, such as mass drug administration (MDA) more feasible [8]. National malaria control programmes that have seen success in malaria control in the last decade are looking to implement new tools to sustain existing reductions and to further reduce transmission. The question is whether primaquine is one of these tools?

Salhiya Ali described current malaria transmission in Zanzibar, which is characterized by perennial and declining transmission. The sporozoite rate decreased from 4.3 in 2005 to 0% in 2009 and the most recent para-site prevalence was 0.067%. Recent Zanzibar Malaria Control Programme reports suggest that transmission has become highly heterogenous with cases restricted to relatively few weeks per year and to a few localities. Primaquine is not used, but its use could be considered to facilitate further reductions by targeting hot spots, or in treating confirmed clinical cases. The local distri-bution of gametocytaemia and G6PD deficiency is not known.

In Ethiopia, both P. falciparum and Plasmodium vivax are endemic and Ashenafi Assefa indicated that the malaria strategy for 2011–2015 includes a plan for elimination by 2020. Primaquine was used in Ethiopia for 25 years up until 1990. Chloroquine (CQ) plus primaquine was first-line treatment for both species. There is no documenta-tion of adverse effects due to primaquine in this period. When sulphadoxine-pyrimethamine (SP) was introduced, it was considered not feasible to administer three drugs, therefore, primaquine was dropped. At present, prima-quine is used for radical cure of P. vivax, but not for P. falciparum. The barriers to using primaquine in Ethiopia include: 1) a lack of documentation of the distri-bution and clustering of G6PD deficiency (small studies suggest that prevalence is between 1.4 and 6.7% among some minority groups) [9], and 2) uncertainty about the efficacy of primaquine for interrupting transmission of P. falciparum in Ethiopia.

Karen Barnes gave a historical perspective of malaria control in South Africa. Previously, the country had high levels of malaria transmission. In 1938, there were 22,000 deaths due to malaria in Kwazulu-Natal. Subsequently, an aggressive approach to malaria control including mapping, malaria surveys, and vector control has reduced the burden considerably but case incidence has remained at a steady state since 2001. The Ministry of Health has now set a goal for elimination by 2018. The biggest chal-lenges include imported malaria, and the perception that malaria is not a public health problem, leading to central budget cuts. Given the already aggressive measures in place, the addition of a transmission-blocking drug such as primaquine could be required to achieve elimination. One challenge is that primaquine is only available on an individual patient basis for radical cure of P. vivax. In

South Africa, the very high rate of tuberculosis and HIV infection means that the potential for drug interactions with other anti-infective therapies must be considered if primaquine is to be used at a population level. The risk of primaquine-associated haemolysis in people living with HIV infection may differ from that in uninfected people.

In contrast to the aforementioned countries, Diadier Diallo reported that malaria transmission in Burkina Faso is still high. The use of a combination of inter-ventions, such as long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS), and effective artemisinin combination therapy (ACT) with a long half-life part-ner drug such as dihydroartemisinin-piperaquine is a proposed strategy. Co-administration of ACT with prima-quine (or alternatives such as methylene blue) for con-firmed malaria episodes and mass drug administration (MDA) may help to further reduce transmission. This strategy may be particularly appropriate in the Sahel area where transmission is highly seasonal and relatively low, making it a potential target for elimination activities. Challenges include the high mobility of human and vec-tor populations particularly from Mali and Niger.

Historical studies on single dose or short course primaquine for blocking transmission of P. falciparum

Chi Eziefula highlighted that the current recommenda-tions for primaquine are based on studies with very small numbers of participants. The parent 8-aminoquinoline, pamaquine (or plasmoquine), developed in the 1920s, was shown to have activity against P. vivax and Plasmodium ovale relapses, and against both sporozoites and gameto-cytes of all species [10,11]. A derivative of pamaquine, primaquine was developed in the 1940s by the United States army to prevent relapse of P. vivax in soldiers returning from Korea and to prevent the import of malaria into the country [12].

In 1973, the WHO recommended a single dose of primaquine (0.75 mg/kg) for malaria transmission-blocking and considered prior screening for G6PD defi-ciency unnecessary [13]. It was not until 2010 that the WHO Malaria Treatment Guidelines (Second Edition) changed to indicate that the risks of haemolysis in G6PD deficient patients should be given consideration prior to primaquine-based interventions.

The currently recommended single dose of primaquine is based on limited efficacy data. In 1961, in Liberia, Burgess and Bray found that a single dose of 0.75-1.5 mg/kg primaquine administered to12 children cleared circulating gametocytes by day 9 [7]. In 1961, also in Liberia, Gunders administered 0.45-1.1 mg/kg of prima-quine in combination with pyrimethamine to 22 children and adults. Gametocytes were cleared after a mean of 5 days post treatment, and no mosquito infections occurred in feeding assays [14]. Primaquine was paired with amodiaquine (AQ) in a large scale MDA conducted by Clyde in 1962 in a hyperendemic area of Tanzania. More than 15,000 subjects were studied in three clusters: weekly administration, fortnightly administration, and monthly administration. Outcome measures included asexual parasite, gametocyte and sporozoite rates. After six months there was a ten-fold reduction in parasite prevalence with weekly and fortnightly administration but not with monthly administration [3]. Except for the work by Clyde, there are no substantial field data that indicate that single dose primaquine decreases trans-mission of P. falciparum.

Safety data for primaquine use in Africa or African Americans are equally limited despite the fact that they inform contemporary guidelines. Burgess and Bray com-ment that primaquine was “well-tolerated”[7]. Clyde reported no safety data and it is unclear who was excluded from treatment [3]. In a series of studies in G6PD deficient African-American volunteers, Alving and colleagues showed that, in three individuals, haemolysis occurred with daily administration of 30mg (approxi-mately 0.5 mg/kg) of primaquine. But, after three weeks, the haematocrit recovered and lower doses resulted in less haemolysis. Eight weekly doses of 60 mg and 45 mg were not associated with haemolysis [15,16]. Daily administration of 30mg of primaquine to African Americans resulted in significant haemolysis in 1%, com-pared to no severe haemolysis when 15 mg was adminis-tered [17]. Tolerance in a pregnant woman (28 weeks gestation) has only been reported by Burgess and Bray, but there was no documentation of birth outcomes [7]. In a more recent study, Kenyan school children were randomized to receive 15mg primaquine daily or three times a week as a malaria prophylactic. It is not clear whether G6PD deficient individuals were included and haemoglobin levels are not reported but again the authors note simply that “primaquine was remarkably well tolerated in our studies” [18].

Kevin Baird remarked that any discussion about primaquine efficacy is necessarily also a discussion about toxicity as there are inherent risks of the drug in situa-tions when the individual patient may not benefit. He highlighted the importance of employing the ethical principles of autonomy, justice and beneficence to game-tocytocidal therapy [19]. The 45 mg dose of primaquine is based on data obtained in very few, healthy indivi-duals. This dose was proposed in an era where the goal of the US military was not to find the lowest efficacious dose, but rather to show that the drug worked. The first dose-finding study by Alving in 1960 included one single patient [16]. It was subsequently observed that daily but not weekly administration of 0.25 mg/lb of body weight (~0.55 mg/kg) to G6PD deficient-children resulted in

haemolysis [20]. Rieckmann and Burgess both showed declines in gametocytes, oocysts and sporozoites following a dose of 45 mg of primaquine but a similar efficacy was seen with lower doses of 30 mg and 15 mg [7,21,22]. Importantly, these evaluations were conducted without co-administration of a blood schizontocidal drug.

In 1944, the US government abandoned pamaquine as a means of preventing relapses of P. vivax due to its haemolytic toxicity and drug interactions. Primaquine was introduced as a gametocytocidal agent at the 45mg dose based on Alving's work, a dose which was readily available and in use for chemoprophylaxis in American soldiers in Southeast Asia at the time. Some significant haemolysis was seen, mostly in African Americans; there were no deaths but there were several cases of renal fail-ure with daily dosing for 14 days[17]. Summarily, the recommended 45 mg dose may be too dangerous for use in mass drug administration, especially given the limited data on transmission reduction with this strategy.

Recent studies on the use of primaquine in Africa

Data from two Tanzanian studies which employed single dose primaquine were reviewed by Teun Bousema. In the first study, treatment with sulphadoxine-pyrimethamine (SP) and artesunate (As) was given to children aged 3 to 15 years with uncomplicated falciparum malaria. They were randomized to receive placebo or a single dose of 0.75 mg/kg of primaquine on the third day of treatment (day 2). Compared to the control arm, primaquine ad-ministration on day 2 decreased the area under the curve of gametocyte density over time and the duration of gametocyte carriage. The effect was apparent for two weeks; using quantitative real time nucleic acid sequence-based amplification (QT NASBA), 3.9% had gametocytes on day 14 in the primaquine arm, and the density was extremely low, compared to a prevalence of 62.7% in the control arm [23]. Haemoglobin fell in both arms but the drop was more pronounced in the primaquine arm. However, this effect was transient and there was no symptomatic anemia. A haemolytic effect was seen even in some individuals without genotypic (A- variant) G6PD deficiency [23].

In a subsequent cluster randomized study, using MDA in lower Moshi [24], single dose primaquine was given with SP plus As treatment to 1110 individuals older than 1 year with primaquine dosages based on weight (approximately 0.75 mg/kg). It was not possible to assess post-intervention incidence or prevalence because P. falciparum transmission had dropped to very low levels. However, safety outcomes, based on haemolysis, were available. Moderate haemolysis occurred follow-ing primaquine treatment in 40% of G6PD deficient (A- genotype) individuals but in only 4.5% of non-deficient individuals. There was no clinical compromise due to anemia in any of the children, except in one child in the primaquine arm, whose haemoglobin dropped from 8.3 g/dL to 4.8 g/dL. It was noted that in all cases haemolysis was transient, recovering by day 14 after treatment.

As a former colleague of Professor Li Guoqiao, Keith Arnold represented him and presented data from an MDA campaign in Moheli Island, Comoros. Dr. Arnold began by reviewing Professor Li's work on primaquine in South East Asia, which served as the basis for the drug regimen used in Comoros. In the late 1990s, Professor Li developed CV8 (320 mg piperaquine phosphate, 32 mg dihydroartemisinin, 5 mg primaquine phosphate, 90 mg trimethoprim). An estimated 1.3 million doses of this drug were administered across Vietnam as part of the National Malaria Control Programme in 2000. There were no documented reports of haemolysis. Data were pre-sented from subsequent dose-finding studies. Artequick (dihydroartemisinin piperaquine given at 0 and 24 hours) was administered in clinical cases followed as inpatients for 30 days followed by administration of 6 mg (7 patients), 7.5 mg (3 patients) or 8 mg (32 patients) of primaquine. A 7.5 mg dose of primaquine rendered gametocytes non infectious at 24 hours. Following 8 mg of primaquine, there were oocysts but no sporozoites in membrane-fed mosquitoes. He decided on the use of Artequick + 9 mg primaquine for MDA after performing safety studies using 8 mg and 10 mg doses in small numbers of indivi-duals with G6PD deficiency in South East Asia. An MDA campaign in 2003 in Cambodia using this regimen resulted in a large reduction in population parasite car-riage over three years [25].

In Moheli Island, Comoros the baseline P. falciparum parasite prevalence in children ranged from 10-95% in 25 villages. Given a mosquito life expectancy of 30 days, the strategy was to give Artequick for three days plus 9 mg of primaquine on day 1 (Round 1) and day 35 (Round 2). Also, beginning on day 21, 9 mg primaquine alone was given every 10 days, 12 times. Patients less than six months of age were excluded. Treatment cover-age for both rounds was reported as >90% and data from monitoring between 2007 and 2009 suggested a reduc-tion of parasite prevalence to <5%. The exception was an area on the south of the island where parasite rates decreased from 94% to 19% with frequent migration from a nearby island suggested as the reason for the persist-ence of parasites. There were no reports of haemolysis, although it was not measured objectively. The baseline prevalence of G6PD deficiency was estimated to be 15%.

G6PD deficiency prevalence testing and safety issues

G6PD is an essential erythrocytic enzyme. G6PD deficiency is one of world's most common genetic polymorphisms. Dennis Shanks described the current array

of diagnostic tests available to test for G6PD deficiency. Testing of the enzymatic activity of G6PD on freshly-collected blood samples is the most widely used method. The NADPH fluorescent spot test is most commonly used and is currently recommended by the International Committee for Standardization in Haematology, but it requires a UV lamp and is difficult to do on high volumes of samples. Other diagnostic tests include cytochemical assays, DNA sequence analysis of the G6PD gene, and some rapid diagnostic test formats not yet validated for public health application. In theory, testing for G6PD deficiency is not difficult, but most tests have limitations for large-scale field application, such as expense, require-ment for electricity, duration of test procedure, and sen-sitivity of reagents to light and heat, low detection threshold, and relatively low throughput capacity.

Rosalind Howes described G6PD deficiency as being widespread in tropical regions of sub-Saharan Africa, commonly affecting over 15% of the male population, and in some isolated areas of West and Central Africa reaching up to 30% of the male population. It is consid-ered that severe G6PD deficiency is likely to exist in Africa but its prevalence is unknown. Shanks noted that country-wide MDA with primaquine has been used in China and Nicaragua, both areas with a low prevalence of G6PD deficiency and that in both programmes there were some cases of severe haemolysis. The three primary safety/ tolerability issues with primaquine are gastro-intestinal upset, methaemoglobinaemia, and haemolytic anemia in those who are G6PD deficient. G6PD enzyme activity is at best a partial biomarker of clinical effect and the clinical effect is likely dependent on other factors including red blood cell count, gender, and other gen-etic factors.

Testing for G6PD deficiency

Gonzalo Domingo observed that genotyping for G6PD deficiency is most commonly carried out for known prevalent mutations at the risk of misclassifying study participants with unknown G6PD deficiency traits as normals. Phenotyping, either quantitative or qualitative, determines G6PD activity in red blood cells and can be defined as a relative deficiency in activity compared to a predefined “normal” activity or in absolute terms in units per gram of haemoglobin. Most studies in Africa have used a semi-quantitative/qualitative fluorescent spot test and observed a high degree of discordance be-tween phenotyping and genotyping not limited to just heterozygous women. Other phenotypic tests e.g. cyto-chemistry can identify heterozygous females. Spectro-photometry is the gold standard and fluorescent spot tests are useful for screening. The ideal specification for a G6PD deficiency test is difficult to achieve as there is no defined acceptable cut-off of G6PD activity. The challenges are that the measurement of enzyme activity is extremely sensitive to temperature, specimen volume, and possibly specimen type. Of the available tests that run on point-of-care platforms, BinaxNOW is limited by its operating temperature and Access Bio by its small sample volume, which may be a source for perform-ance variability. The BinaxNOW test detects a cut-off of 30-40% enzyme activity and was designed to detect hemizygous males. Detecting heterozygous females re-quire platforms that can detect and enumerate intra-erythrocytic G6PD activity. The next steps include an evaluation of currently available tests for G6PD defi-ciency under ideal laboratory conditions, field evaluation under controlled conditions, and engaging with the diagnostic sector to define a value proposition for point-of-care G6PD deficiency tests. Ongoing efficacy studies for primaquine represent an opportunity to obtain G6PD deficiency cut-off levels.

Examples of possible study designs— clinical and field-based

Lorenz von Seidlein and Teun Bousema considered the sequence of studies required to establish the role of primaquine in the response to artemisinin resistance as well as for the elimination of falciparum malaria. Before population-level interventions are considered, three main questions will need to be addressed: 1) What drug con-centration is needed to inhibit gametocytes, 2) which primaquine regimen is required to achieve these gameto-cyte inhibitory concentrations and 3) can this dose be safely administered to both sexes and all age groups? Excluding young children and women of reproductive age from MDA will seriously reduce coverage and is likely to render any intervention meaningless. Since a prospective study of giving single dose primaquine dur-ing pregnancy is not likely to be approved, retrospective approaches e.g. pharmacovigilance during large field trials should be explored as a way of gaining informa-tion about the safety of primaquine in pregnancy.

One option for field evaluation is the cluster rando-mized trial. A double-blinded community-randomized, placebo-controlled trial in The Gambia evaluated MDA with sulphadoxine- pyrimethamine (SP) plus single dose artesunate (AS1) in 18 villages and achieved 89% coverage [26]. There was an initial decrease in malaria incidence but the effect quickly disappeared. Possible reasons for a failure to reduce transmission intensity might be that the baseline transmission intensity was too high, that there was migration of infected individuals or mosqui-toes, or that the drug regimen was not ideal. A double-blinded community-randomized, placebo-controlled trial was conducted in Tanzania in a setting of very low and

seasonal malaria transmission (entomological inoculation rate of approximately 2) using MDA with SP on day 1 plus artesunate for 3 days and primaquine on day 3 [27]. Coverage of 93% was achieved, but the study failed to show a reduction in transmission intensity due to the small number of outcome events (P. falciparum infections) in both the intervention and control groups. These studies raise two important questions: 1) Are sub-microscopic parasite densities sufficient to sustain transmission and

2) what is the ideal transmission intensity at which to conduct MDA? It was considered that studies designed to detect the community benefit of ACT versus ACT plus primaquine would potentially necessitate very large sample sizes and alternative strategies to evaluate MDA should also be considered. The community effect of insecticide-treated bed nets extends beyond the households that use nets and has been estimated by measuring the distance between control and intervention villages and com-pounds where protection is seen. Such an effect may exist for primaquine- based interventions such that targeted coverage has a high impact. Less ambitious trial designs could encompass treatment of clinical malaria cases, focal screen–and-treat campaigns, or primaquine could be incorporated into active case detection activities using standardized outcome measures such as entomological parameters, gametocyte prevalence by molecular methods, parasite prevalence/ molecular force of infection, and malaria incidence during follow-up.

The design of trials of MDA with primaquine should help inform a potential strategy for interventions. What is the threshold endemicity level at which MDA with primaquine should be considered? How many rounds of MDA are required and at what interval to give a given effect? Even if efficacy and safety can be established, the issue of willingness to participate in MDA must be con-sidered. In settings of very low transmission and minimal risk, e.g. Swaziland, the community might not be as accepting of MDA as compared to a country with higher endemicity as the perceived benefit is lower.

Potential study endpoints-clinical and field studies

Heiner Grueninger emphasized that study endpoints should be designed to facilitate both effective treatment and increased knowledge of the study drug. In the con-text of using primaquine for a new indication of trans-mission-blocking, the study design should address the requirements set by authorities for obtaining regulatory approval to use the drug. Consequently, endpoints should be considered with input from both industry and policy makers in order to expedite drug deployment in endemic settings.

Chris Drakeley discussed biomedical efficacy end-points. Abrogation of infection in mosquito infectivity studies is a compelling functional bioassay yet only one existing study involving primaquine satisfied Cochrane review criteria (Graves and Gelband, in press). In this study, mefloquine and SP plus primaquine stopped infection over 14 days post treatment [28]. The mosquito feeding assay methods for assessing post treatment infectivity of subjects offer different options for evaluation, but are not standardized. Direct skin feeding of mosquitoes on treated individuals is most representative of natural infection dynamics but presents logistical and ethical concerns. Using venous blood allows both direct membrane feeding but also serum replacement with untreated or treated serum to examine the effect of dif-ferent serum compositions, such as drug metabolites. Reproducibility of results is an important issue with no clear guidelines on how to feed mosquitoes, how many mosquitoes should be fed per assay, because the robustness of the estimate of prevalence of infection depends on the number fed [29], and on which day post-treatment participants should be tested for infectivity. For example, primaquine has a short half-life so infectivity could be measured after 24 hours, whereas, for the purpose of MDA, it is probably pertinent to know for how long the subject has reduced infectivity and testing for infectivity up to 28 days may be relevant. This latter point could be addressed by staggering sampling time points between participants to reduce the number of bleeds per individ-ual. Further studies may be required to confirm the effect on infectiousness to wild mosquito populations as natural infections have been shown to be successful at very low gametocyte densities suggesting high vector susceptibility [30]. Such feeding experiments may not be warranted or practical for larger field evaluations and a surrogate marker for transmission would be preferable.

Although, there is no standardized, validated marker of infectiousness of the human host, the most widely used marker to compare drugs is the prevalence of gameto-cytes 7 days post treatment [31]. Gametocyte density is less relevant at low gametocyte counts found in chronic and asymptomatic infections as the correlation between infectivity and low gametocyte density is poor. The measurement of gametocyte prevalence and density depends on the method of detection with 5- to 10- fold differences seen with molecular methods compared to microscopy[32]. Gametocyte densities can be integrated using area under the curve (AUC) to provide an estimate of gametocyte carriage [33,34]. In natural infections this is likely to vary by age with young children with clinical disease having short, intense gametocytaemia (abrogated by drugs or gametocyte death) and older semi-immune individuals, who can have asymptomatic infections for up to a year [35] and maybe longer, with a more pro-longed AUC.

The issue of how to tailor the design of studies using primaquine to include endpoints that are meaningful to

regulatory authorities was tackled by Justin Green. The key question is what level of evidence do we require in order to use primaquine as a transmission-blocking agent? He referred to ongoing studies using tafenoquine to high-light how bespoke endpoints are being used to achieve licensure. Tafenoquine is an 8-aminoquinoline developed by the US army and the Walter Reed Army Institute of Research (WRAIR) with GlaxoSmithKline (GSK). It has a long half-life (14–17 days), which may confer advan-tages as an anti-parasitic agent, but also risks, given that the duration of haemolysis in individuals with G6PD deficiency is also prolonged [36]. The drug is slowly metabolized and the parent compound is respon-sible for the anti-malarial effect [37]. Tafenoquine is being developed as a radical cure of P. vivax infection. Green described a dose-ranging study in individuals over 16 years with P. vivax infection evaluating chloro-quine alone compared with standard dose chloroquine plus primaquine 15mg (for 14 days) and different single doses of tafenoquine (50mg, 100mg, 300mg, and 600mg) given on day 1 or day 2 (NCT01376167). The primary endpoint is relapse at 6 months with secondary end-points of relapse at 4 months, time to relapse, parasite clearance time, fever clearance time, gametocyte clear-ance time (by microscopy), safety and pharmacokinetics/ pharmacodynamics.

These pivotal endpoints are designed with regulatory requirements in mind so that wording related to end-points can be incorporated into a label claim. From the perspective of industry, this can determine the potential volume of sales (the percentage of the primaquine market obtainable). For trials with primaquine, or other transmission-blocking candidates, it is necessary to decide how important it is that the study endpoint is on the label and whether stakeholders demand a “label claim” or an indication for approval. For transmission markers to stand as endpoints for a regulatory level trial, one would need validation that the marker, e.g., a

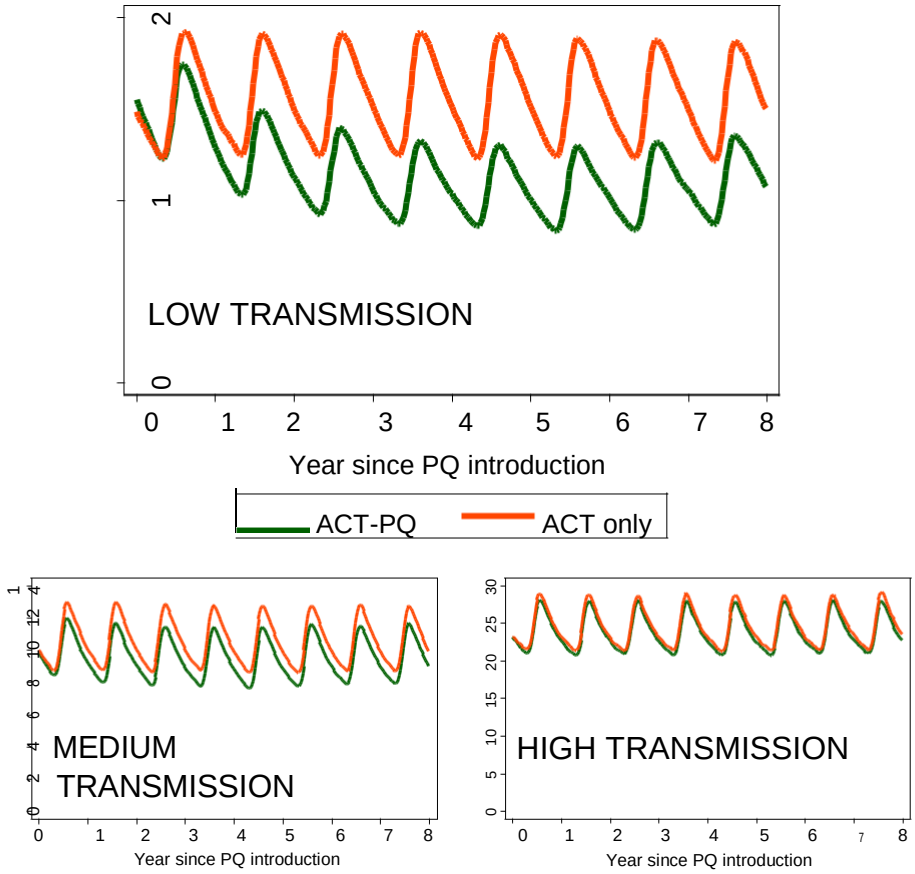

Figure 1 Preliminary modeling of administration of primaquine together with ACTs in a range of transmission settings. A simulation of adding primaquine to ACT first line treatment versus ACTs only in a seasonal setting. In this simulation 80% of clinical cases are treated with ACT alone or ACT-PQ. Primaquine is assumed to reduce the duration of infection by 78% and the level of infectiousness by 67% in treated patients compared to those treated with ACT alone. In a low transmission scenario, adding PQ to the treatment of clinical cases causes a higher relative reduction than in higher transmission scenarios. Ro differs between settings. Migration is not allowed for. With the kind permission of Lucy Okell, Jamie Griffin & Azra Ghani. For further details, see reference [40].

molecular method such as detection of pfs25 with QT NASBA [38] or microscopic gametocytaemia, correlates with transmission.

Typically, the pharmaceutical industry focuses on the risk-benefit of a particular drug in the individual. For primaquine, the drug may be of more benefit to some-one other than the recipient, raising the ethical question of whether it is acceptable to give a drug for community benefit. This issue is also pertinent to transmission-blocking vaccines [39]. Justin Green considered that it is crucial that primaquine trials include individuals with G6PD deficiency (including heterozygote females) and describe the risk of haemolysis in these patients. There is no consensus on whether there is any acceptable degree of haemolysis following a drug intervention for malaria in clinical cases or in asymptomatic individuals.

Modeling the potential use of primaquine

Teun Bousema discussed how to extrapolate the effect of primaquine in the individual to community-level transmission, acknowledging that the infectious reservoir of malaria may vary with transmission setting. A recent model by Lucy Okell and colleagues [33] suggests that infectiousness post ACT alone is 13 days and post ACT plus primaquine is 3 days. Using this model that incorporates population age structure, immunity, heterogeneous exposure and as well multiple interventions as covariates, the addition of primaquine to ACT as first-line treatment significantly reduces transmission in low endemic set-tings but not in higher transmission settings (Figure 1). The proportion of people who received primaquine in addition to ACT is a key parameter suggesting prima-quine needs to be given with all courses of ACT to have an effect. The models were further extended to investi-gate the effect of primaquine as part of an MDA [40] in a non-seasonal setting with 9% prevalence of P. falciparum. Giving MDA every four months caused an 80% reduction in transmission, but not elimination. With MDA every six weeks one could plausibly reach elimination. Prelim-inary models suggest that MDA may be more successful in areas of seasonal transmission (Figure 2). The dur-ation of drug action is important and a long acting ACT plus a long acting 8-aminoquinoline could be an optimal combination.

An approach targeting malaria transmission hotspots may be appropriate for all endemic settings [8]. The hypothesis is that hot spots catalyse transmission and targeting them would reduce transmission both within and outside the hotspot. Modeling hotspot interventions with no drug treatment but with insecticide-treated bed nets scaled up to 80% coverage and targeted IRS had a significant effect on transmission. The effect of adding primaquine should be investigated. Models of transmis-sion assume a long time-course and there was discussion

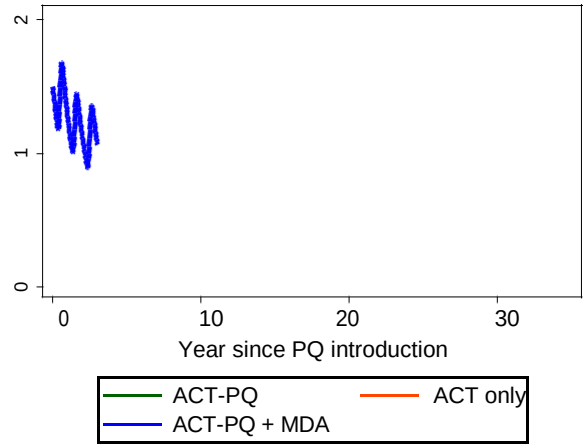

Figure 2 The effect of annual MDA with primaquine in addition to primaquine and ACT treatment of clinical cases in a low transmission setting. In a seasonal, low transmission setting, giving ACT+ primaquine to clinical cases plus an annual MDA with ACT plus primaquine could reduce malaria levels close to elimination if repeated for a number of years; however the model does not allow for immigration of malaria cases. With the kind permission of Lucy Okell, Jamie Griffin & Azra Ghani. For further details, see reference [40].

as to the stability of hot spots and how this would affect the efficacy of an intervention.

Meeting outputs

Possible approaches for the use of primaquine to interrupt malaria transmission

Having reviewed the existing data, the second aim of the meeting was to identify the roadblocks to deployment of primaquine in Africa (Figure 3), decide on common study endpoints and to determine the next steps. As a starting point the group determined the intended indica-tions of primaquine (Figure 4), a target product profile (Figure 5) and common endpoints for infectivity, efficacy and safety studies (Figure 6).

Key roadblocks to the deployment of primaquine

Safety and efficacy of primaquine

The paucity of evidence for primaquine's safety and efficacy for transmission-blocking were seen as major issues, particularly, the lack of data supporting the safest and most efficacious dose. If primaquine is going to be used to maximal benefit then it must be safe to deploy in G6PD deficient individuals and women of childbearing age and it must be safe to co-administer with HIV and tuberculosis treatments without adverse drug interactions. Most crucially, evidence is lacking for any transmission-reducing effect in the community from the addition of

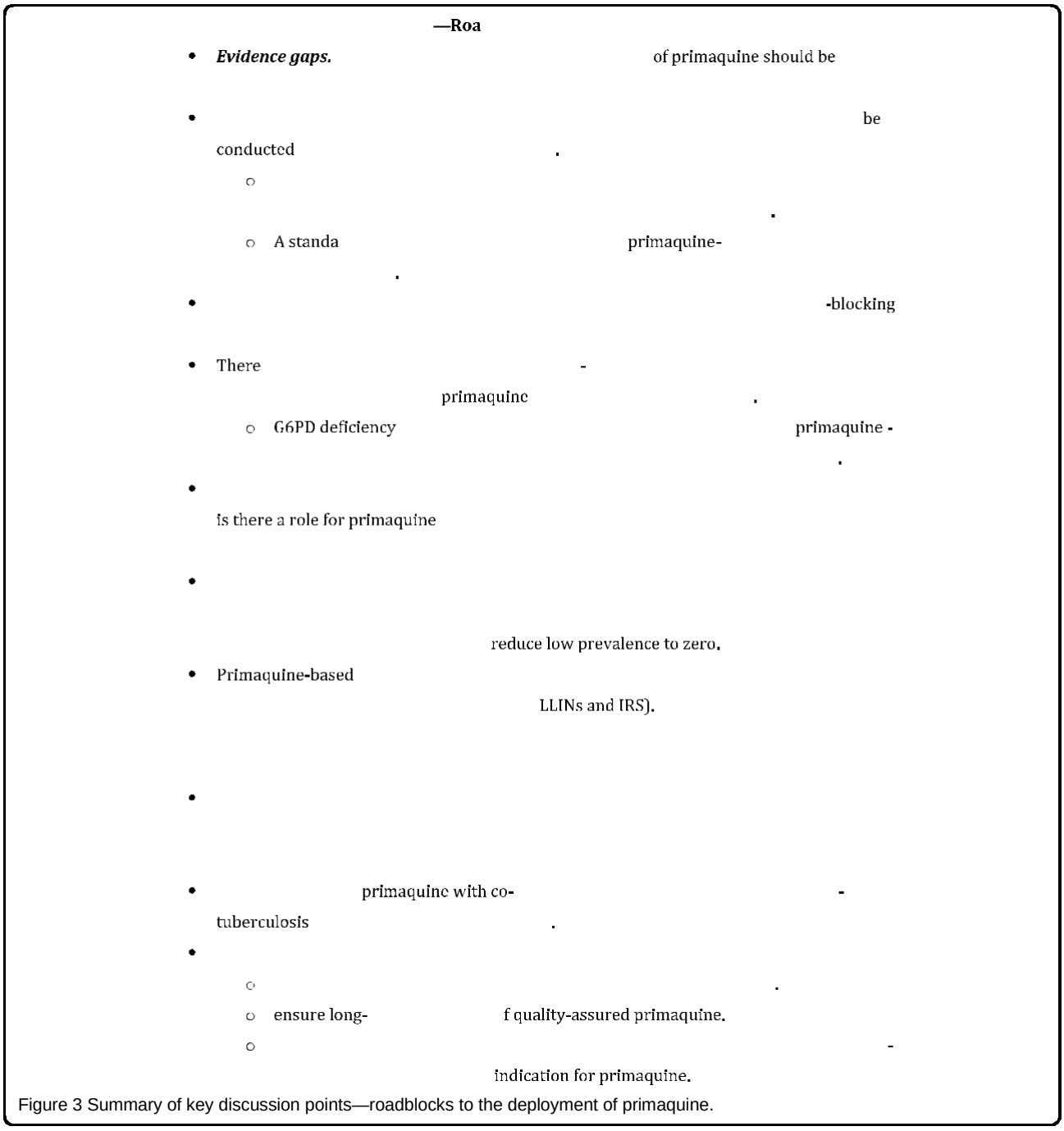

Figure 3 Summary of key discussion points—roadblocks to the deployment of primaquine.

primaquine to routine anti-malarial treatment of symptomatic individuals.

Suitable endemicity for use of primaquine

It was agreed that use of primaquine is most likely to have an impact on transmission intensity in areas char-acterized by low endemicity prior to the intervention, i.e. P. falciparum parasite rate (PfPR) by microscopy of less than 5%, or an EIR (entomological inoculation rate) less than 1. In such settings, there is a low frequency of symptomatic parasitaemia so the greatest benefit is likely to result from treating asymptomatic infections as well, through MDA or screen-and-treat initiatives. The optimal strategy for delivering primaquine-based MDA in terms of who to treat, at what threshold endemicity, with what regimen and how often is unknown.

Mathematical modelling indicates a limited effect at higher transmission intensities (PfPR> 10%). However,

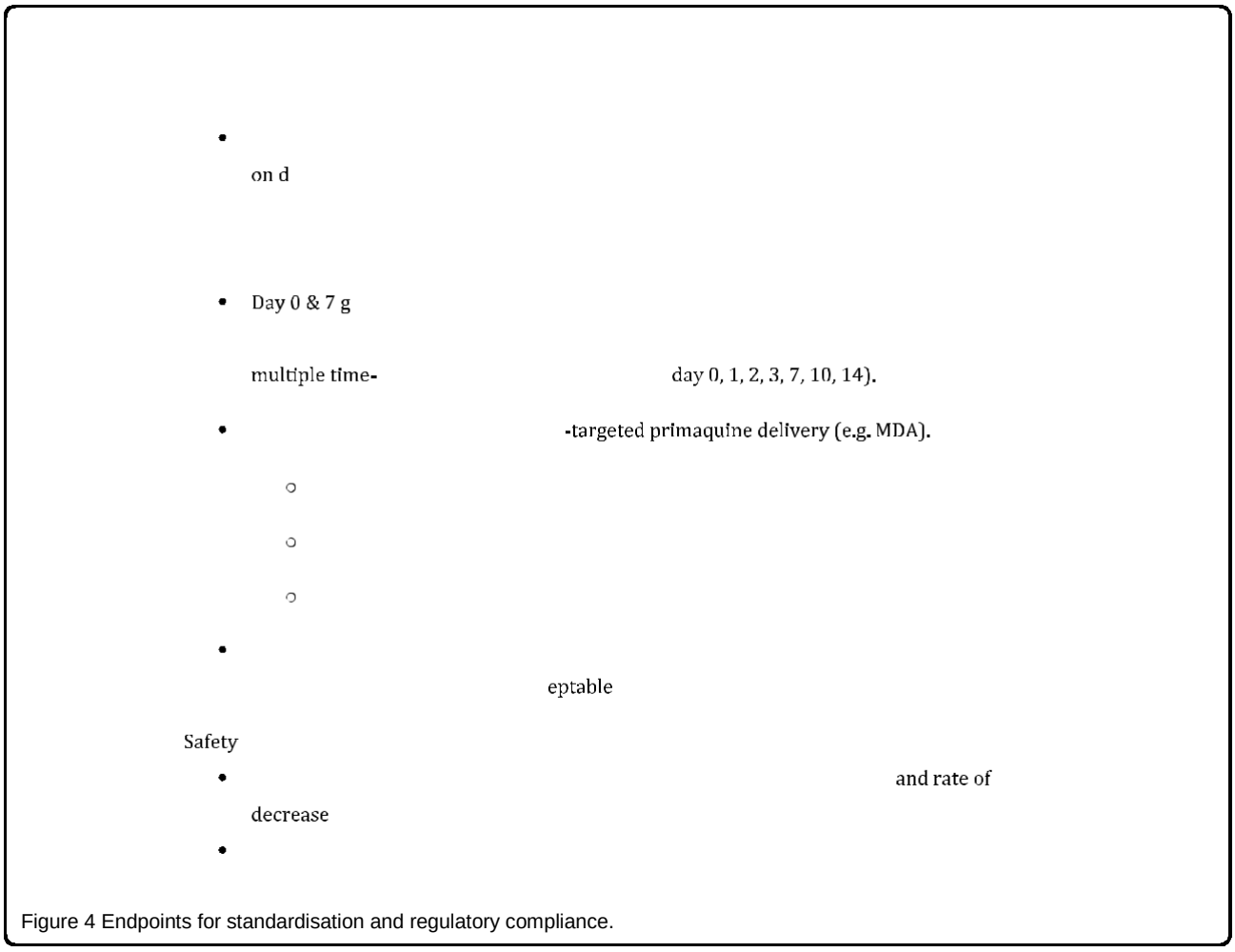

Figure 4 Endpoints for standardisation and regulatory compliance.

further iterations are needed to assess the additional effect of primaquine interventions together with other control tools at a range of transmission intensities. As was the situation in Aneityum, Vanuatu [41], there may be other higher transmission settings where interruption of transmission could occur using MDA with primaquine because of limited human migration.

Partner drug for primaquine

For community campaigns with primaquine, the partner ACT should probably differ from the recommended first-line anti-malarial treatment. An alternative ACT may be required for community-wide MDA or in circumstances where repeated rounds of MDA are envisaged. However, in smaller hotspots of high transmission intensity where fewer rounds of treatment with ACT-primaquine are needed, the standard first-line ACT could be considered as the partner to primaquine. The relative gametocytocidal activity of the partner ACT, its half-life for killing asexual parasites and the potential for drug interactions or for synergy with primaquine will need to be considered.

Drug supply and regulation

The manufacture and supply of the appropriate dose and formulation of primaquine was seen as a major obstacle for primaquine deployment. Currently, there are primaquine shortages globally and in Africa, the procurement of supplies to treat P. vivax where it is endemic is a challenge.

Further information on the current challenges for the manufacture and supply of single- or low-dose prima-quine is required. A review of the current situation of primaquine manufacture and supply should be carried out with the aim of identifying the steps needed to ensure an adequate supply of primaquine formulated in the correct dose should low dose primaquine be found to be efficacious. It is likely that primaquine for the clear-ance of P. falciparum gametocytes will remain off label. In order to ensure the smooth process from manufacture to implementation, it was recommended that stake-holders from industry and governments, including regu-latory authorities be brought together to discuss these challenges.

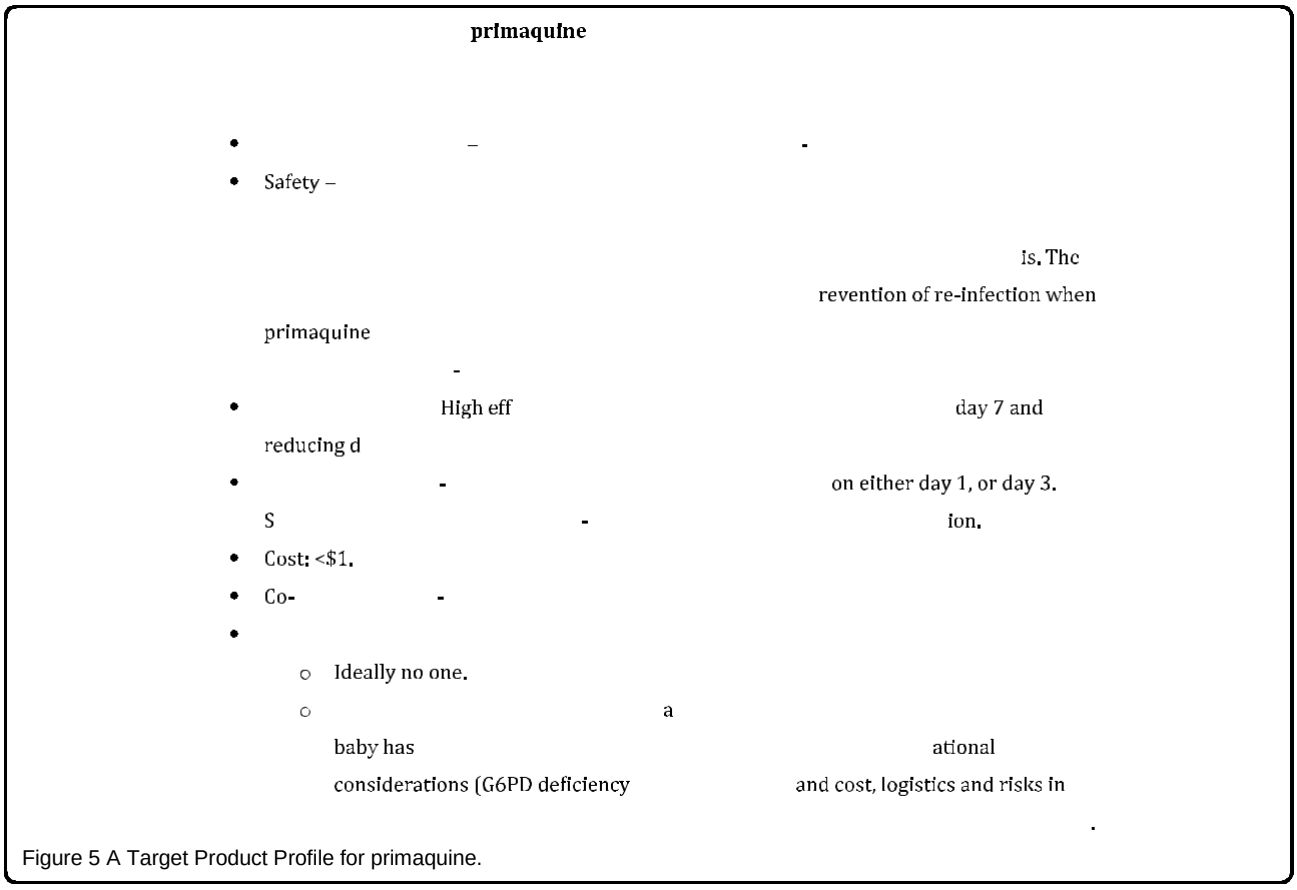

Figure 5 A Target Product Profile for primaquine.

Alternatives to primaquine

The meeting agreed that seeking alternative gametocytoci-dal drugs to primaquine was paramount due to the safety concerns with 8-aminoquinolines. The 8-aminoquinoline tafenoquine appears to have a similar safety profile to primaquine (haemolysis in people with G6PD deficiency), but being long-acting, may potentially inhibit gametocyte infectivity for longer. Should a safe, low dose be found, tafenoquine could be a useful tool in the elimination of P. falciparum. There is increasing evidence for methy-lene blue having a better safety profile [42,43], but more work needs to be done on regimen, dose-finding and acceptability [44,45]. The group supported the further development of these drugs and considers it a priority to develop more compounds active against transmission stages for all species of malaria.

The roadmap

Three themes were identified that need to be addressed simultaneously. Firstly, there are evidence gaps for primaquine itself, secondly, the manufacture and supply of primaquine needs mapping and thirdly, efforts to search for a safe and effective alternative to primaquine need to be supported. A schematic of the roadmap is shown in Figure 7.

Providing evidence of the efficacy and safety of primaquine

A range of studies from phase 1–4 were proposed that would inform decisions on the efficacy and safety of primaquine. These are outlined below.

Phase 1: Identification of the lowest dose for efficacy

Ex vivo gametocytocidal/ infectivity assays: because the active metabolites of primaquine are currently unknown, the interpretation of in vitro assays with primaquine is complicated. A possible approach would be to use healthy volunteers treated with different doses of primaquine. The plasma (containing primaquine metabolites) of these individuals could be used in membrane feeding experi-ments with cultured parasites to demonstrate lack of infectivity in mosquitoes of different doses of primaquine and in combination with ACT.

There is no proven relationship between mosquito infectivity and gametocytocidal effects so this may need to be repeated with a variety of parasite lines and volun-teers of different ethnic backgrounds.

It was noted that much needed pharmacokinetic studies could be performed during the same experiments as could studies evaluating the different partner ACT, other gametocytocidal drugs and drugs in common use

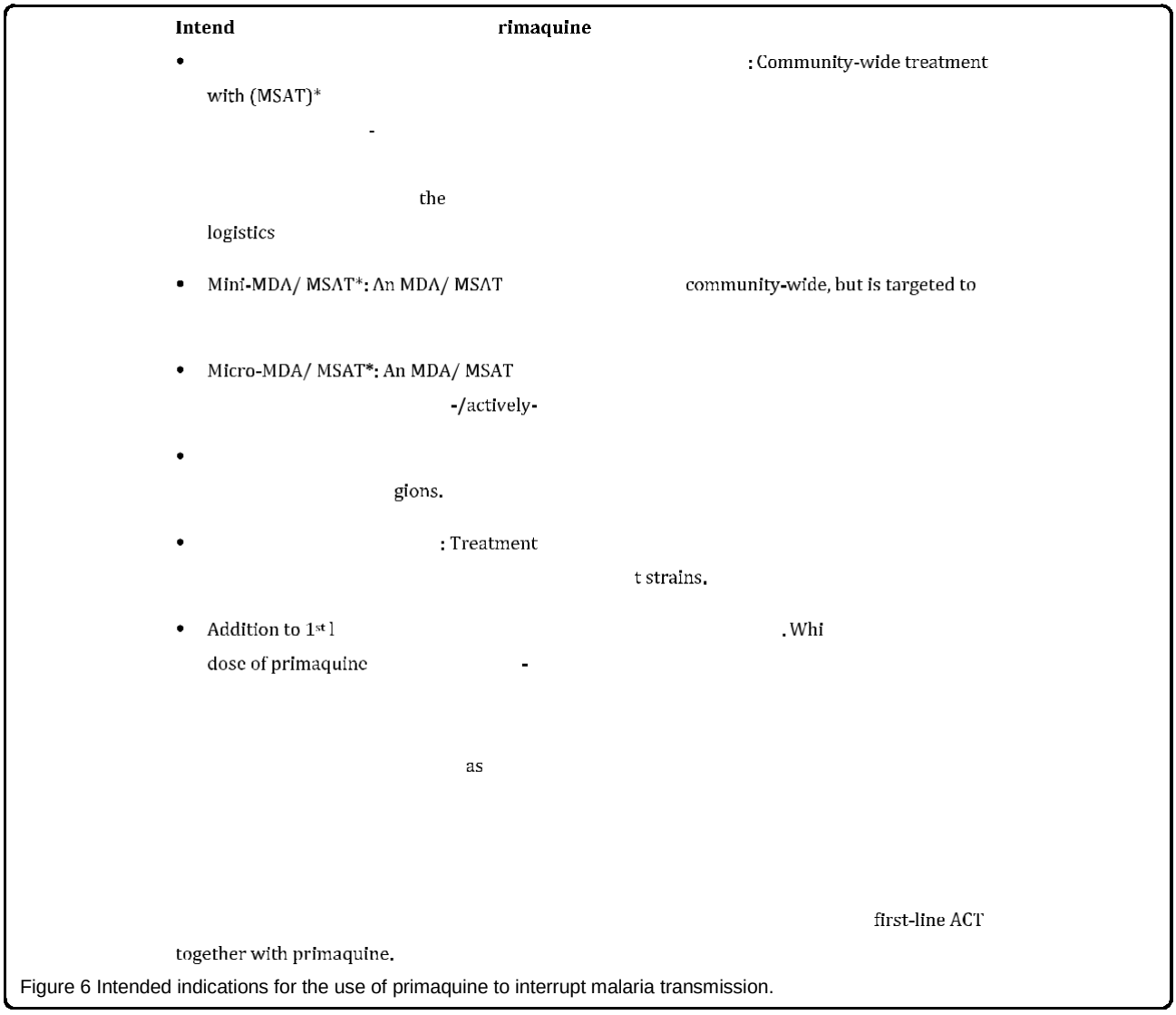

Figure 6 Intended indications for the use of primaquine to interrupt malaria transmission.

that may interact with primaquine (e.g. antiretrovirals and drugs for tuberculosis).

Phase 2: Establish the safety and efficacy of the optimal dose of primaquine in relevant sub-groups

Efficacy of low dose primaquine to assess post-treatment infectivity using common endpoints (see below) in G6PD normal individuals. A dose-finding study is currently under way in Uganda (NCT01365598).

Studies to confirm safety of low dose primaquine in G6PD deficient.

– hemizygous males with lowest doses (dose escalation studies)
– heterozygous females (dose escalation studies)
– individuals of a given phenotypic G6PD enzyme function level, to establish a relationship between G6PD enzyme function level and safety, a proposed threshold enzyme function being in the range 20-30%.

Confirm safety and efficacy in infected population of unselected G6PD status (timeline 3–4 years).

If safety with G6PD deficiency remains a problem, field usable and reliable point of care tests to detect G6PD deficiency will be needed and the effect of not treating a proportion of the population on transmission reduction modeled.

Programmes to map the geographical distribution of G6PD deficiency in countries targeted for primaquine deployment. This should include assessment of the range of enzyme function levels in the population.

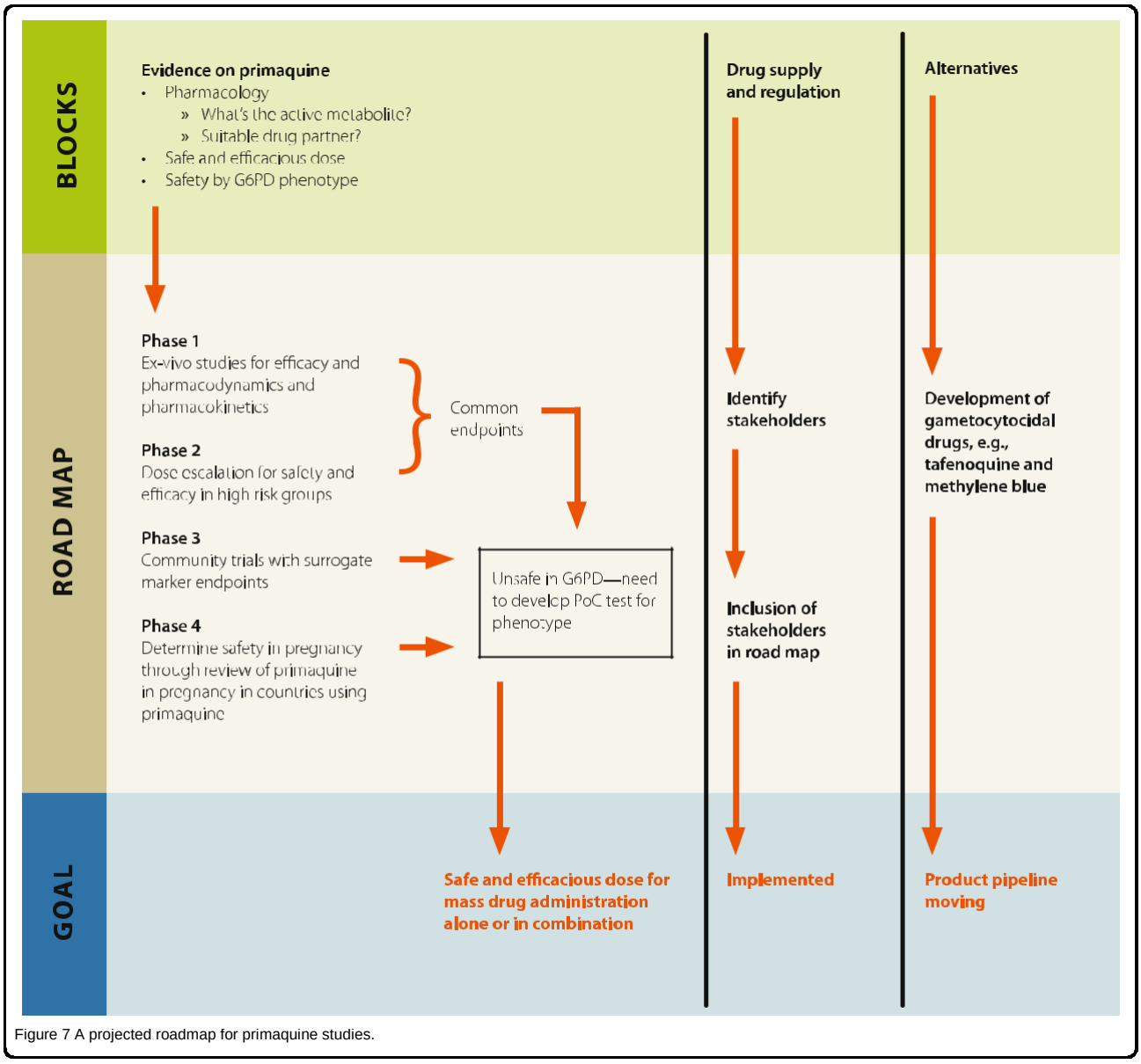

Figure 7 A projected roadmap for primaquine studies.

Phase 3: Studies to establish utility at community level pharmacovigilance could be supported to do a retro-These may measure transmission reduction but may not spective study following up women of reproductive age necessarily need to be in the form of randomized con- who have been treated with any dose of primaquine. trolled trials. Much can be learnt from the transmission-blocking vaccine field where designs such as a 'stepped wedge' design may be used with a focus on the indirect and community effects. Both prospective and retrospective pharmacovigilance studies will be needed and pregnancy registers will be an important component.

Phase 4: Studies to review the safety in pregnancy
Currently there is no evidence on safety of primaquine in pregnancy. Post-marketing surveillance is possible as several countries have adopted primaquine as policy, such as India, China and Sri Lanka. In these countries,

Conclusion

Primaquine may be a useful malaria control tool in low-endemic settings in Africa when used in combination with a blood schizontocide. For maximal effect it will need to be given to asymptomatic parasite carriers and therefore a safe and efficacious dose needs to be found that can be used in populations with G6PD deficiency. Studies designed to find this dose should contain common endpoints including infectiousness to mosquitoes seven days after treatment and gametocyte prevalence pre-treatment and seven days post-treatment to allow

maximal comparability between trials. Safety endpoints need to be defined, particularly with regard to G6PD pheno- and genotype and pregnancy. Methylene blue and tafenoquine are alternative drugs but need further testing and establishing standard protocols could facili-tate this process. Community trials should identify the added benefit of using primaquine in addition to a long-acting ACT with the endpoint of community transmis-sion reduction.

Abbreviations
ACT: Artemisinin combination therapy; As: Artesunate; AUC: Area under the curve of gametocyte density over time; CQ: Chloroquine; EIR: Entomological inoculation rate; G6PD: Glucose-6-phosphate dehydrogenase; IRS: Indoor residual spraying; LLINs: Long-lasting insecticidal nets; MDA: Mass drug administration; PfPR: P. falciparum parasite prevalence; PQ: Primaquine; QT-NASBA: Quantitative real time nucleic acid sequence-based; SAE: Severe adverse event; SP: Sulphadoxine-pyrimethamine; WHO: World Health Organization.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ACE, RG and CD wrote and edited the manuscript, ACE, MH and JH were meeting rapporteurs and together with TB and LvS, contributed to the manuscript. All authors read and approved the final manuscript.

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2.4 Trialling an off-label drug

An important challenge in setting up this trial was the fact that primaquine was not on the registered drugs list in Uganda (244) and was not part of the treatment policy for the Ugandan National Malaria Programme. Therefore, it was not manufactured in the country and needed to be imported for use in the trial. This raised several issues surrounding the off label use of the drug.

2.4.1 What is on primaquine's label and why does it matter?

A drug's label defines the indications for which the drug has been found in clinical trials to be safe and effective (245). Primaquine appears on the WHO Essential Medicines list for the indication of radical cure of *Plasmodium vivax* infection at doses of 7.5mg or 15mg (246) and this is a reflection of the label it is approved for across different drug authorities (247). The use of single, low-dose primaquine to block transmission of *Plasmodium falciparum* infection is an off-label use of the drug.

There are numerous examples where off-label drug use has come into common practice and is even recognised as the first-line treatment option (248). It is legal for clinicians to prescribe off-label drugs once they are on the market, as long as it is "done in good faith, in the best interest of the patient and without fraudulent intent" (249).

2.4.2 Licence to trial an off-label drug

Primaquine had not been used in Uganda for the transmission-blocking indication previously and there were no other African countries implementing it as a gametocytocide for malaria control or elimination at the time of this trial. Approval from the Ugandan National Drug Authority for the importation of the drug and its use in a trial use necessitated the application for a clinical trial licence. This involved documentation of the chemistry of the product, the proposed non-Ugandan manufacturer and evidence of their quality control processes, and summaries of non-clinical and clinical studies conducted on the pharmacokinetics, safety and

efficacy of the drug, and post-marketing experience. The full clinical trial licence application for the national drug authority is available in Appendix B. Table 2-7 summarises its key contents:

Table 2-7 Ugandan National Drug Authority clinical trial licence application checklist

Requirements for NDA Clinical Trial Licence application
☐ Proof of payment of fees ☐ Materials transfer: Applications for import and/or export of materials ☐ Clinical Trial Application Form ☐ Trial Protocol ☐ Investigators Brochure ☐ Participant Information Leaflet and Informed Consent ☐ Certificate of GMP manufacture of the trial medicine or other evidence of manufacture quality, safety and consistency ☐ Package Insert/s for other trial medicines. ☐ Certificate of GMP manufacture of the placebo - if appropriate. ☐ Evidence of accreditation of the designated Laboratories or other evidence of GLP and assay validation. ☐ Insurance Certificate specific for the trial in consultation with NDA ☐ Signed and completed Declarations by all Investigators ☐ Approval of Ethics Committees for the Protocol ☐ Full, legible copies of key, peer-reviewed published articles supporting the application. ☐ Sample of the label for the imported products

2.4.3 The challenge of ensuring accurate paediatric dosing of primaquine

Primaquine is available in tablets containing 15mg and 7.5mg primaquine phosphate base. For the purpose of this dose-finding trial in children, doses as small as 1mg, increasing in increments of 0.5 mg, were required. It was necessary to develop a robust method for incremental dosing that would ensure the correct amount of active drug in each dose administered.

The stability of primaquine phosphate tablets in solution with water was established by HPLC analysis in the laboratory of Dr Harparkash Kaur at the London School of Hygiene and Tropical Medicine. The analysis demonstrated that primaquine tablets, including the batch procured for the study were stable in solution at room temperature (20 degrees C) for 7 days. This meant that doses could be titrated accurately by syringe for the purpose of the clinical trial. Each tablet of 15mg primaquine phosphate was crushed and fully dissolved in 15ml of drinking water. This 1mg/ml solution was used to give draw up doses to the nearest 0.5mg using 5ml and 10ml syringes.

This methodology was subsequently ratified in 2014 (two years after this trial completed), when Sanofi Aventis produced instructions for the extemporaneous preparation of primaquine phosphate tablets 26.3mg (15mg equivalent base) for clinical trial use. These instructions were shared at the low dose primaquine working group meeting and published in the meeting report (250).

2.5 Ethical clearance and regulation

2.5.1 Ethical committee assessment

The trial was submitted to ethical committees at the London School of Hygiene and Tropical Medicine in the UK, to the Faculty of Medicine Research Ethics Committee (FOMREC) at

Makerere University College of Health Sciences, Uganda and to the Ugandan National Council of Science and Technology (UNCST).

2.5.1.1 *Ethical recommendations from Faculty of Medicine Research Ethics Committee (FOMREC) at Makerere University College of Health Sciences, Uganda*

The ethical committee did not express concern with the ethics of the principle of using primaquine as a transmission-blockier and assessing the dose-response relationship. There was recognition that its use was recommended in the WHO guidelines. Given the contra-indication in pregnancy, concerns were raised regarding the method of exclusion of pregnancy. The committee agreed that limiting the eligible age limit to 10 years, and excluding those who have started menstruating, was sufficient, but at their request, a supply of pregnancy tests was provided at the study clinic, to be used at the discretion of the study clinicians and with parental consent.

FOMREC requested simplification of the language in the consent forms. This prompted consultation with local researchers and community members with experience in constructing and using consent forms in the study village location, where many participants had a limited extent of formal education.

Detail of quality assurance systems was provided on request, particularly on how malaria slides would be read and how results would be validated. This had been detailed in the study protocol (Appendix A) (200). There was a call for specification with regard to future use of biological specimens, as it was thought to be too broad in the initial protocol version. Accordingly, this was narrowed to cover only research related to malaria.

The appropriateness of cost re-imbursements for participants was questioned, and justification was given detailing the expected costs incurred by participants for food and transport in missing a day of work.

The committee was interested to learn what was the intended system for clinical management of illnesses other than malaria, and it was confirmed that all diseases would be managed within the capacity of local facilities and expertise and any requirement for specialist medical input would be met by consultation with dedicated senior clinicians at the regional paediatric hospital (which was 30 minutes' drive away).

There was concern with regard to the optimisation of opportunities for capacity building by processing study samples within Uganda as far as possible. Unfortunately, potential for this was limited, as at the time of study development and with the available resources, the technology for molecular gametocyte detection and pharmacokinetic analysis specifically for primaquine were not available in Uganda. Initially, polymerase chain reaction detection of G6PD alleles was planned in country, but unfortunately, the infrastructure was not available in a timely manner and these samples were instead exported, under appropriate transport conditions, for analysis in the UK. Malaria microscopy, haemoglobin measurement, G6PD phenotypic assessment (fluorescent spot test) and G6PD enzyme level assays were all conducted in Uganda.

2.5.1.2 *Ethical recommendations from London School of Hygiene and Tropical Medicine (LSHTM)*

The first proposal submitted to LSHTM (and all ethical committees) involved a trial population that was un-screened for G6PD deficiency. This was in line with the lack of requirement for G6PD screening in the WHO recommendations for single dose primaquine. The proposal was rejected outright by the LSHTM ethical committee, with the following comment:

"We are of the opinion that the benefit to the individual participants from the primaquine is small if not negligible and the risk relatively high. We therefore do not feel able to approve the proposal as submitted. There are clearly possibilities for staging this work - with a dose

ranging study in those screened to ensure they are not G6PD deficient followed by a study of the safety of this dose in the general population."

This feedback was considered extremely helpful in highlighting the expected ethical concerns and the trial design was altered to exclude G6PD deficient children at enrolment. Plans were put in place to develop a protocol for a daughter trial in G6PD deficient individuals informed by the dosing data in this trial (251).

The new trial population demanded a re-calculation of the appropriate sample size. Initially, sample size was calculated to include adequate numbers to assess safety in the predicted proportion of individuals who would be G6PD deficient. Upon revision, the optimal sample size for safety was reduced, as there was no longer any requirement to include a population that would be expected to represent all G6PD genotypes. As no prior representative data was available on the expected fall in haemoglobin in a G6PD-screened African population, data was taken from a recent trial of primaquine in an un-screened population in Tanzania (63) . The revised sample size calculation takes into account the size required for non-inferiority analysis of the efficacy outcome measure in the test dose arms compared to the reference dose (WHO-dose) arms and the size required to assess the superiority of safety outcome measures in the test dose arms compared to the reference dose (WHO-dose) arm.

The process of screening for G6PD deficiency was not specified by the committee. Specific issues with the selection of the method for G6PD screening are covered in Section 1.2.3.6.

2.5.1.3 *Ethical recommendations from the Uganda National Council of Science and Technology (UNCST)*

No concerns were raised at the stage of protocol approval by the UNCST, but, following their site visit to inspect the acceptability of the trial site prior to recruitment, recommendations were made regarding the storage facilities for study drugs and the appropriateness of snacks

and provision of meals for study participants and their relatives whilst attending for follow up visits.

2.5.2 Clinical trial registration, sponsorship and monitoring

The trial was registered on www.clnicaltrials.gov. The trial was sponsored by LSHTM, and LSHTM conducted the clinical trial monitoring. All monitoring site visits were conducted by the Ugandan National Council of Science and Technology (UNCST).

A Data Safety and Monitoring Board (DSMB) was set up for the trial. All members accepted the terms of a DSMB charter. The board agreed what data and parameters should be presented and at what frequency. Data collated per study arm was coded as A, B, C and D to preserve blindness. A sample DSMB reporting sheet is available in Appendix C, (part 1).

2.6 Site set up

2.6.1 Collaboration with the Infectious Diseases Research Collaboration, Uganda

The collaborator in Uganda was the Infectious Diseases Research Collaboration (IDRC), a non-profit research organisation that was established in May 2008 by scientists at Makerere University College of Health Sciences and the University of California, San Francisco and the Ugandan Ministry of Health.

The IDRC evolved from the Uganda Malaria Surveillance Project established in 2001 to evaluate the impact of malaria control interventions on key malaria indicators. This focussed on sentinel sites based at six health centres across the country. The organisation's role expanded rapidly to incorporate the production of high quality data from multiple geographical sites and research programmes targeted to inform policy makers and widened research activities in the fields of HIV, tuberculosis and community health as well as malaria.

Key components of collaboration with the IDRC were support for Ugandan trial regulatory affairs, including communication with the ethical committees, and provision of training for staff to ensure compliance with Good Clinical Practice requirements; support with data management, including provision of a secure server to deposit trial data; quality control of malaria slide readings at the Kampala-based Molecular Research laboratory (MOLAB); support with procurement, budget administration, staff employment and financial reporting; and, assistance with sample transport and storage facilities

This work saw the involvement of the London School of Hygiene and Tropical Medicine as a new collaborator with IDRC, necessitating the set-up of a Memorandum of Understanding between the two organisations, to facilitate exchange of contracts and funds and transfer of materials for the purpose of this study and future work.

2.6.2 Blood transfusion access

Access to safe blood transfusion was vital for this study in the event of any severe drug-related haemolysis. An agreement was made with the Regional Paediatric Referral Hospital in Jinja to provide blood (form the national bank in Kampala) to our study participants. The regional paediatric intensive care unit clinicians agreed to support our study participants if they required blood transfusion.

2.6.3 Medical care infrastructures

At the study site, the resident governmental health workers were not medically qualified. Two qualified physicians were employed for the trial as study co-ordinators and doctors. The lead paediatrician at the Regional Paediatric Referral Hospital in Jinja provided senior support to

the trial physicians when requested. Details of the senior consultations requested by trial staff are provided in the results section (Section 3.3.3).

2.6.4 Staff recruitment and training

Study staff were recruited and employed through IDRC-hosted structures. Group and role-specific training sessions were designed to cover all trial processes. All staff were trained and certified in Good Clinical Practice (GCP) and materials for Good Clinical and Laboratory Practice (GCLP) were supported by the IDRC and a series of group discussion sessions were held in the laboratory to further explore the training manual. The data team had input into the design of a self-cleaning study database. Briefly, the database was designed so that each value had to fit appropriate criteria in order to be entered. This reduced some level of human data entry error. Manual data cleaning was still required, hence all data was double entered and checked for inconsistency and corrected against source data before being entered into the master database. The laboratory team had input into the design of some of the laboratory standard operating procedures (SOPs). The clinicians, nurses and fieldworkers also inputted into SOP design after training sessions to provide guidance. This helped ensure that staff members had ownership of the targets and processes of the clinical trial.

Training for each staff discipline was given by the principle investigator at employment and trial-specific training manuals were designed and provided. Materials on the ethics of research involving human subjects developed by the collaborating partner, IDRC, were used for additional group training in this area. These covered a history of research ethics, recognition of vulnerable populations, key elements of the informed consent process and guidelines on correct documentation of informed consent. All staff had permanent access to these materials for continued reference during the recruitment process.

Once trial recruitment was underway, there were regular appraisal sessions and feedback opportunities for staff to assess performance, to improve practice where necessary and to give feedback to the principle investigator and employer.

2.7 Overcoming hurdles

2.7.1 Delays in obtaining permissions

The initial submission of the study protocol to the School of Medicine Research Ethics Committee at Makerere University, Kampala, was not reviewed because the committee announced that it was undergoing a change in staffing and a change in processes. During this time, the submitted protocol was reported to have been lost from the committee offices. This was discovered only after some months of waiting for approval, adding to delays. The protocol was re-submitted and then reviewed, after which, revisions were called for as detailed above (Section 2.5.1) and the submitted alterations were approved. At the point of protocol alteration, core trial staff were appointed and trained, in order to minimise further delays in the onset of recruitment, whilst balancing the cost of staff employment prior to approval.

2.7.2 Delays during recruitment—widened catchment area

The incidence of clinical malaria at the study site had been recorded by the Uganda Malaria Surveillance Project showed an expected blood slide positivity rate of approximately 50% (252). This had been used to predict the rate of recruitment and to plan the study landmarks and budget. The rate of recruitment to the trial underwent significant decline after the first two months. Data were obtained on the seasonal rainfall in the region and this was not lower than expected (253). Estimates of the entomological inoculation rate (number of infective bites per person per year) from epidemiological surveillance projects at the study site revealed significant reduction from 7 (254) to 3.8 (255) over the preceding 7 years.

Given the budgetary consequences of a reduced recruitment rate, the decision was taken to widen the catchment area for the study. This decision was taken in consultation with ethical advisers from the collaborating partner, IDRC. Participants were screened at health centres and hospitals within the widened catchment area and were then transported by study staff (always less than 20 minutes' travel) to the study clinic, where screening processes were repeated and eligible individuals were invited to enter the enrolment process. They then completed the final steps of screening at the study clinic and were consented if entry criteria were satisfied. All of their subsequent follow up visits were conducted at the study clinic.

3 Results: Primaquine safety and efficacy dose-finding trial

This chapter covers the results of the trial, the main findings of which were published in the peer-reviewed Lancet Infectious Diseases journal (256). Additional data analysis considerations and unpublished data are presented in sections 3.2 to 3.3. Immediately following the trial, the methodology and results were shared in international meetings to develop and progress the research agenda and to engage with policy makers. These data sharing processes are described here in Section 3.3.4.

3.1 RESEARCH PAPER 3: Publication of trial results

The trial results were peer reviewed and published in the Lancet Infectious Diseases Journal. See Appendix E for the accepted plain text version of the manuscript.

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RESEARCH PAPER COVER SHEET

Please note that a cover sheet must be completed for each research paper included within a thesis.

SECTION A – Student Details

Student ID Number	257918/RITD	**Title**	Dr
First Name(s)	Alice Chijioke		
Surname/Family Name	Eziefula		
Thesis Title	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Lancet Infectious Diseases		
When was the work published?	13th November 2013		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
Have you retained the copyright for the work?*	**No**	Was the work subject to academic peer review?	**Yes**

*If yes, please attach evidence of retention. If no, or if the work is being included in its published format, please attach evidence of permission from the copyright holder (publisher or other author) to include this work.

SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	
Please list the paper's authors in the intended authorship order:	
Stage of publication	Choose an item.

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the study together with TB, SY, NJW, ELW, SGS and CD and I conducted the data interpretation and writing together with TB, SY, NJW, ELW, SGS and CD. I implemented and led the study. All authors reviewed and approved the final version.

SECTION E

Student Signature	Chi Eziefula [redacted]
Date	18th September 2019

Supervisor Signature	
Date	23rd September 2019

Single dose primaquine for clearance of *Plasmodium falciparum* gametocytes in children with uncomplicated malaria in Uganda: a randomised, controlled, double-blind, dose-ranging trial

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Summary

Background **Primaquine is the only available drug that clears mature *Plasmodium falciparum* gametocytes in infected human hosts, thereby preventing transmission of malaria to mosquitoes. However, concerns about dose-dependent haemolysis in people with glucose-6-phosphate dehydrogenase (G6PD) deficiencies have limited its use. We assessed the dose-response association of single-dose primaquine for gametocyte clearance and for safety in *P falciparum* malaria.**

Methods **We undertook this randomised, double-blind, placebo-controlled trial with four parallel groups in Jinja district, eastern Uganda. We randomly allocated Ugandan children aged 1–10 years with uncomplicated falciparum malaria and normal G6PD enzyme function to receive artemether–lumefantrine, combined with either placebo or with 0·1 mg/kg, 0·4 mg/kg, or 0·75 mg/kg (WHO reference dose) primaquine base. Randomisation was done with computer-generated four-digit treatment assignment codes allocated to random dose groups in block sizes of 16. Study staff who provided care or assessed outcomes and the participants remained masked to the intervention group after assignment. The primary efficacy endpoint was the non-inferiority of the mean duration of gametocyte carriage in the test doses compared with the reference group of 0·75 mg primaquine per kg, with a non-inferiority margin of 2·5 days. The primary safety endpoint was the superiority of the arithmetic mean maximum decrease in haemoglobin concentration from enrolment to day 28 of follow-up in the primaquine treatment groups compared with placebo, with use of significance testing of pairwise comparisons with a cutoff of p=0·05. The trial is registered with ClinicalTrials.gov, number NCT01365598.**

Findings **We randomly allocated 468 participants to receive artemether–lumefantrine combined with placebo (119 children) or with 0·1 mg/kg (116), 0·4 mg/kg (116), or 0·75 mg/kg (117) primaquine base. The mean duration of gametocyte carriage was 6·6 days (95% CI 5·3–7·8) in the 0·75 mg/kg reference group, 6·3 days (5·1–7·5) in the 0·4 mg/kg primaquine group (p=0·74), 8·0 days (6·6–9·4) in the 0·1 mg/kg primaquine group (p=0·14), and 12·4 days (9·9–15·0) in the placebo group (p<0·0001). No children showed evidence of treatment-related haemolysis, and the mean maximum decrease in haemoglobin concentration was not associated with the dose of primaquine received—it did not differ significantly compared with placebo (10·7 g/L, SD 11·1) in the 0·1 mg/kg (11·4 g/L, 9·4; p=0·61), 0·4 mg/kg (11·3 g/L, 10·0; p=0·67), or 0·75 mg/kg (12·7 g/L, 8·2; p=0·11) primaquine groups.**

Interpretation **We conclude that 0·4 mg/kg primaquine has similar gametocytocidal efficacy to the reference 0·75 mg/kg primaquine dose, but a dose of 0·1 mg/kg was inconclusive for non-inferiority. Our findings call for the prioritisation of further trials into the efficacy and safety of doses of primaquine between 0·1 mg/kg and 0·4 mg/kg (including the dose of 0·25 mg/kg recently recommended by WHO), in view of the potential for widespread use of the drug to block malaria transmission.**

Funding **Wellcome Trust and the Bill & Melinda Gates Foundation.**

Introduction

Effective drug therapy is a key component of malaria control and elimination strategies to reduce both morbidity from the disease and onward transmission to mosquitoes.[1] Artemisinin combination therapy (ACT), the first-line treatment in sub-Saharan Africa, achieves excellent cure rates for *Plasmodium falciparum* through rapid clearance of the asexual stages of the parasite. As a consequence, ACT reduces the production of malaria transmission stages—gametocytes—and thereby restricts transmission potential.[2] However, onward malaria transmission is not completely prevented because of the inadequate effect of artemisinins and their partner drugs against mature gametocytes. If mature gametocytes are present before treatment, they persist after ACT, often at concentrations below the threshold for detection by conventional microscopy,[3] and can allow onward malaria transmission for up to 14 days after treatment.[3–6]

Primaquine, an 8-aminoquinoline, is the only available drug with established activity against mature gametocytes. It clears circulating gametocytes that persist after ACT, thereby reducing the duration of gametocyte carriage,[7–12] and renders most patients free of gametocytes by day 14 after initiation of ACT–primaquine treat-ment.[7–9,12] Primaquine reduces the transmission of malaria to mosquitoes—an effect that might precede the clearance of gametocytes.[13,14] The transmission-blocking properties of primaquine have been reviewed in detail.[15] WHO has recommended one dose of primaquine in addition to ACTs for use in two scenarios: for malaria elimination programmes, and to stop the spread of emerging artemisinin resistance.[16] Primaquine is recommended for use in first-line antimalarial treatment in many countries.[17]

Despite these recommendations, primaquine is often not used because of concerns about its haemolytic effect in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Primaquine-induced haemolysis can occur after one dose of the drug[18] and is dose dependent.[19] Because doses of primaquine lower than the WHO-recommended dose can be equally efficacious at clearance of *P falciparum* gametocytes,[15] dose optimisation for ACT–primaquine is needed.

No formal randomised controlled trials have been done to characterise the dose-response relation of primaquine for *P falciparum* gametocyte clearance. We aimed to assess the efficacy of reduced doses of primaquine for non-inferiority to the WHO reference dose of 0·75 mg primaquine base per kg that has proven efficacy,[7,20] and to assess for superiority of the safety of reduced doses compared with placebo, in people with normal G6PD enzyme function.

Methods

Study design and participants

The study was a randomised, double-blind, placebo-controlled trial with four parallel groups. The study protocol has been described in detail elsewhere.[21] Briefly, we undertook the study at Walukuba Health Centre IV in Jinja district, eastern Uganda, between December, 2011, and March, 2013. In this region, malaria transmission is perennial with seasonal peaks in intensity. An entomological inoculation rate of seven infectious bites per person per year was estimated in 2001.[22]

Eligible participants were children aged 1–10 years attending the health centre with fever or history of fever in the past 24 h, *P falciparum* monoinfection with a parasite density lower than 500 000 per μL, and normal G6PD enzyme function based on a fluorescence spot test (R&D Diagnostics, Aghia Paraskevi, Greece). Exclusion criteria were evidence of severe illness or danger signs, haemoglobin concentration less than 80 g/L, known allergy to the study drugs, antimalarials taken within the past 2 days, primaquine taken within the past 4 weeks, and blood transfusion within the past 90 days. Written informed consent was provided by parents or guardians and, in addition, assent was provided by children older than 8 years of age.

Ethics approval for the trial protocol and informed consent forms were provided by the Makerere University School of Medicine research ethics committee (protocol 2011-210), the Uganda National Council of Science and Technology (protocol HS1056), and the London School of Hygiene and Tropical Medicine research ethics committee (protocol 5987). The Ugandan National Drug Authority approved importation of the study drug. The trial data safety monitoring board and trial advisory committee were convened before the start of the trial and met at predetermined stages of the study. Consultations with local community stakeholders in Walukuba were held before, during, and after trial completion.

Randomisation and masking

We randomly assigned eligible participants to one of four dose groups. In each group, we gave participants artemether–lumefantrine twice daily on days 0–2 and, with the fifth dose of the drug, one dose of either placebo or primaquine (0·1 mg/kg, 0·4 mg/kg, or 0·75 mg/kg). A statistician at the London School of Hygiene and Tropical Medicine (ELW) computer-generated four-digit treatment assignment codes and allocated these to random dose groups in block sizes of 16. To achieve treatment concealment, we added masking syrup to all treatment groups, which disguised the colour and taste of the study drug. Because G6PD deficiency is an X chromosome-linked disorder, we stratified randomisation by sex. Sequential sealed envelopes containing a randomisation code were selected by the study pharmacist from either the male or female pile. The pharmacist was not involved in patient outcome assessment. All other study staff providing care or assessing outcomes, and the participants themselves, remained masked to the intervention group after assignment.

Procedures

We crushed 15 mg base primaquine phosphate tablets and dissolved them in 15 mL of drinking water to produce a stable 1 mg/mL solution. We drew up the assigned dose to the nearest 0·5 mL through a sterile syringe and immediately gave it to each participant in a plastic cup or spoon. We administered all treatments after the children had eaten a fatty snack (biscuits) and then directly observed the patients. If a child vomited within 30 min, treatment was re-administered. Those who vomited more than three times were excluded from the study and were treated for complicated malaria.

Enrolled participants were reviewed on days 0, 1, 2, 3, 7, 10, 14, 21, and 28, or on additional days if they presented at the clinic. We did systematic and prospective assess-ments for adverse events. We graded new or worsening symptoms, examination findings, or laboratory ab - normalities according to a severity scale (adapted from

the WHO toxicity grading scale for determining the severity of adverse events and from the National Institutes of Health, Division of Microbiology and Infectious Diseases paediatric toxicity tables published in January, 2003)[23] and assessed causal associations with the study drug. We implemented a standardised protocol to detect episodes of haemolytic anaemia, which we have published elsewhere.[21] On scheduled visits, we collected roughly 500 μL of venous blood for laboratory assess-ments. On all visits, we did asexual malaria parasite counts, in which we enumerated parasites per 200 white blood cells; we read 100 microscopy fields in the Giemsa-stained thick blood film before we judged a slide to be parasite negative. At enrolment, we read slides twice specifically for gametocytes, following the same procedure as that for asexual parasites. We measured haemoglobin concentration on days 0, 1, 2, 3, 7, 10, 14, 21, and 28 with self-calibrating HemoCue 201+ photometers (HemoCue; Angelholm, Sweden). We assessed gameto-cytaemia by quantitative real-time nucleic acid sequence-based analysis (QT-NASBA) with *Pfs25* mRNA[24] on days 0, 2, 3, 7, 10, and 14. The timing of gametocytaemia measurements was based on findings from previous studies that suggested the gametocyte-clearing effect of primaquine is restricted to the first 2 weeks after treatment.[7,25] We extracted nucleic acids from 50 μL blood samples in L6 buffer (Severn Biotech Limited, Kidderminster, UK) with Total Nucleic Acid Isolation Kits–High Performance (Roche Applied Science, Mannheim, Germany) and a MagNA Pure LC automated extractor (Roche Applied Science). The sensitivity of this assay is related to the volume of blood sampled and is in the range of 0·02–0·1 gametocytes per μL for the samples collected.[24]

The primary endpoint for efficacy was the non-inferiority of the mean duration of gametocyte carriage in the test doses compared with the reference group of 0·75 mg primaquine base per kg. Secondary endpoints were the point prevalence of gametocytes on days 7, 10, and 14 after treatment, gametocyte circulation time, and the area under the curve (AUC) of gametocyte density over time after primaquine administration. For treatment outcomes in each group, definitions of adequate clinical and parasitological response, early treatment failure, and late treatment failure were according to WHO Methods for Surveillance of Antimalarial Drug Efficacy.[26] The primary safety endpoint was the superiority of the arithmetic mean maximum decrease in haemoglobin concentration from enrolment to day 28 of follow-up in the primaquine treatment groups compared with the placebo group. Secondary safety endpoints were the superiority assessment of the day of haemoglobin nadir, the maximum percentage decrease in haemoglobin, the percentage of participants with haemoglobin concentration lower than 50 g/L, requirement for blood transfusion, evidence of black urine, and the frequency of severe adverse events.

Statistical analysis

In our sample size calculation, we took into consideration the primary endpoints for both efficacy and safety. To guide the efficacy calculation, we used the QT-NASBA-measured duration of gametocyte carriage in a Tanzanian study, which was reduced from a mean of 28·6 to 6·3 days (SD 6) when primaquine (0·75 mg/kg) was added to ACT alone.[25] Efficacy analyses were done on an intention-to-treat basis. To assess non-inferiority of the test groups to the reference group with 80% power at the two-tailed 5% significance level, with allowance for 10% loss to follow-up and with use of a proposed clinically relevant non-inferiority margin of 2·5 days, the target sample size for efficacy was 120 participants per group. However, during the course of review by the trial data safety monitoring board, the target sample size was reduced to 460 participants (ie, 115 per group instead of 120) because of a lower than expected loss to follow-up. For the safety component of our analysis, the sample size calculation was based on the mean decrease in Hemocue-measured haemoglobin concentration on day 7 after treatment with primaquine of 6 g/L (SD 15) in a previous Tanzanian study.[18] A sample size of 99 participants per group would provide 80% power to detect a difference in mean maximum decrease in haemoglobin between treatment groups of 6 g/L at a significance level of 5%.

Data were double entered and transferred into Stata (version 12.0) for analysis. We estimated duration of gametocyte carriage and gametocyte circulation time in children with gametocytaemia on day 2 (the day of primaquine dosing) with a straightforward deterministic compartmental mathematical model[25] that allows for the release of gametocytes from sequestration and incorporates baseline gametocyte densities into model estimates. The model allows the duration of gametocyte carriage to be estimated as a continuous outcome. As the spacing between sampling times increases, some uncertainty is expected, but this was judged to be acceptable for estimates during the first 14 days after initiation of treatment. We compared treatment groups for non-inferiority to the reference group with two-sided 95% CIs. Because the distribution of gametocyte densities was expected to be skewed, all density analyses involved log10-transformed data and we used geometric means as summary statistics. We assessed the AUC of gametocyte density per participant with the linear trapezoid method[27] and log10-transformed the data. We used ANOVA to compare log AUC with the reference treatment group. We compared gametocyte point prevalence estimates per treatment group with the reference group with use of the prevalence ratio with 95% CIs. We adjusted all efficacy analyses for gametocyte density at enrolment, and tested the potential effect of sex by adding this variable to multivariate models and by doing a stratified analysis.

The primary safety outcome, maximum decrease in haemoglobin (g/L) during follow-up compared with the measurement at enrolment, is expressed as an arithmetic

mean per treatment group and pairwise comparisons made between placebo and each of the primaquine groups, with unpaired *t* tests. We used a cutoff for significance tests of p=0·05 for the superiority analysis. We compared the occurrence of adverse events between groups; the significance level was adjusted for several comparisons by Bonferroni correction. This trial is registered with ClinicalTrials.gov, number NCT01365598.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study. All authors reviewed the report and agreed to submit for publication.

Results

We screened 1215 children with a history of fever and a positive blood smear at Walukuba Health Centre for eligibility to enrol in the study. The most frequent reason for exclusion was having taken antimalarial drugs in the previous 48 h (figure 1). Between December, 2011, and December, 2012, we enrolled and randomly allocated 468 children, 461 of whom completed treatment and contributed data for the assessment of safety and efficacy (figure 1). 36 of these 461 children (8%) did not complete 28 day follow-up. The proportion lost to follow-up did not differ significantly between treatment groups, but was highest in the placebo group (figure 1). Baseline characteristics were similar in all treatment groups (table 1). 199 of 461 (43%) children were anaemic at baseline (haemoglobin concentration <110 g/L). Treatment failure, assessed clinically and microscopically, was rare (table 2) and did not differ significantly between groups (p=0·68).

Gametocyte prevalence at enrolment was 22·6% (104/461) by microscopy and 81·8% (365/446) by QT-NASBA (table 1), and did not differ between treatment groups (p=0·91 for microscopy and p=0·42 for QT-NASBA). Gametocyte density at enrolment was numerically higher in the 0·75 mg/kg reference group (table 1) but did not differ significantly from any of the other groups (p≥0·31). Gametocyte prevalence decreased

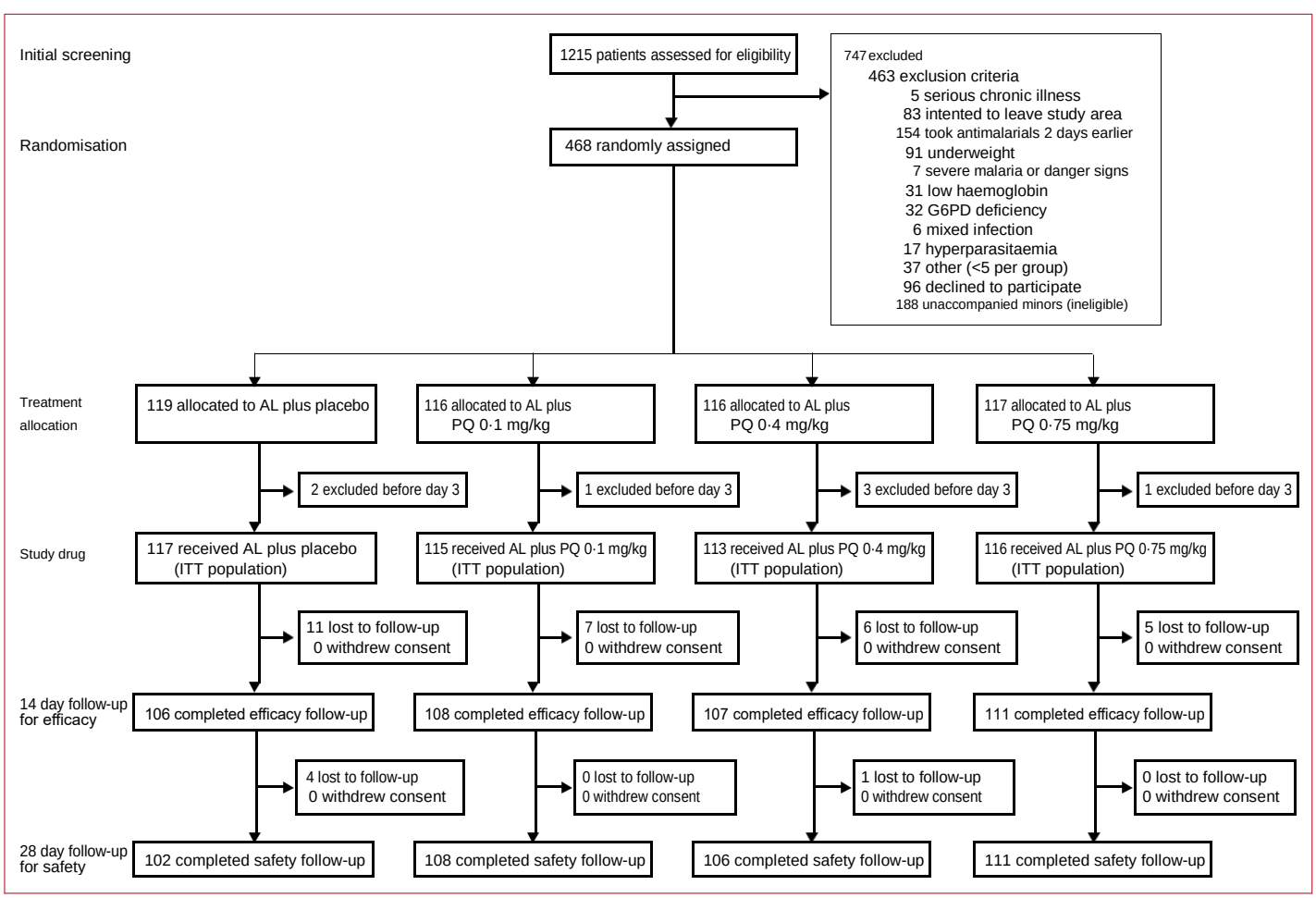

Figure 1: **Trial profile**

AL was given as six doses over 3 days (days 0, 1, and 2); PQ or placebo was given together with the fifth dose of AL on the morning of day 2. The two post-treatment exclusions in the 0·4 mg/kg treament group (because of delayed confirmation of parasitaemia) were followed up for safety. G6PD=glucose-6-phosphate dehydrogenase. AL=artemether–lumefantrine. PQ=primaquine. ITT=intention to treat.

	Placebo (n=117)	Primaquine 0·1 mg/kg (n=115)	Primaquine 0·4 mg/kg (n=113)	Primaquine 0·75 mg/kg (n=116)
Boys	48·7% (57/117)	49·6% (57/115)	49·6% (56/113)	49·1% (57/116)
Age (years)	5·0 (3·0–7·5)	5·0 (3·3–7·0)	5·3 (3·2–7·0)	4·1 (3·0–7·0)
Bodyweight (kg)	16·0 (13·0–20·5)	16·0 (13·0–22·0)	17·0 (14·0–23·0)	15·0 (13·0–19·0)
Body temperature (°C)	38·0 (1·0)	38·3 (1·1)	38·0 (1·2)	38·2 (1·1)
Haemoglobin concentration (g/L)	113 (15)	109 (15)	112 (15)	112 (14)
Geometric mean sexual parasite density, parasites/mL (IQR)	17 661 (5260–65 130)	18 420 (4440–92 780)	16 457 (3260–81 240)	32 497 (10 880–151 180)
Gametocyte prevalence by microscopy	23·1% (27/117)	24·3% (28/115)	20·4% (23/113)	22·4% (26/116)
Gametocyte prevalence by QT-NASBA	79·8% (91/114)	86·7% (98/113)	78·7% (85/108)	82·0% (91/111)
Geometric mean gametocyte density (gametocytes/μL) by QT-NASBA (IQR)	15·2 (8·4–27·8)	14·5 (8·9–23·5)	19·4 (11·3–33·1)	24·6 (14·9–40·5)

Data are % (n/N), median (IQR), or mean (SD), unless otherwise indicated. QT-NASBA=quantitative real-time nucleic acid sequence-based analysis.

Table 1: **Baseline characteristics**

	Placebo	Primaquine 0·1 mg/kg	p value*	Primaquine 0·4 mg/kg	p value*	Primaquine 0·75 mg/kg	p value*
Number evaluated	117	115	..	113	..	116	..
Excluded from ITT analysis							
Withdrawal unrelated to study drug or malaria	0	0	..	2/113 (1·8%)	0·245	0	..
Lost to follow-up	15/117 (12·8%)	7/115 (6·1%)	0·080	7/113 (6·2%)	0·088	5/116 (4·3%)	0·033
ACPR on day 28	98/102 (96·1%)	101/108 (93·5%)	0·41	106/106 (100%)	0·12	106/111 (95·5%)	0·83
Treatment failures							
Early (day 3)	0	0	..	0	..	0	..
Late (day 28)	4/102 (3·9%)	7/108 (6·5%)	0·41	0	0·12	5/111 (4·5%)	0·83

Data are n/N (%), unless otherwise indicated. ITT=intention to treat. ACPR=adequate clinical and parasitological response. Defi nitions of ACPR, early treatment failure, and late treatment failure are according to WHO Methods for Surveillance of Antimalarial Drug Efficacy 2009.[26] *p values are for comparison with placebo, with χ^2 or Fisher's exact tests. Outcomes are unadjusted by PCR.

Table 2: **Treatment outcomes for the different regimens on day 28 after start of treatment**

	Placebo	p value*	Primaquine 0·1 mg/kg	p value*	Primaquine 0·4 mg/kg	p value*	Primaquine 0·75 mg/kg
Duration of gametocyte carriage (days)†	12·4 (9·9–15·0)	<0·0001	8·0 (6·6–9·4)	0·14	6·3 (5·1–7·5)	0·74	6·6 (5·3–7·8)
Circulation time per gametocyte (days)	1·97 (1·64–2·31)	<0·0001	1·47 (1·22–1·73)	0·0012	0·95 (0·77–1·13)	0·80	0·98 (0·78–1·18)
Gametocyte prevalence on day 7	40/115 (34·8%)	0·001	25/108 (23·1%)	0·044	11/104 (10·6%)	0·47	15/104 (14·4%)
Gametocyte prevalence on day 10	23/112 (20·5%)	0·008	18/107 (16·8%)	0·020	10/107 (9·3%)	0·46	8/108 (7·4%)
Gametocyte prevalence on day 14	16/105 (15·2%)	0·017	6/103 (5·8%)	0·72	3/103 (2·9%)	0·51	6/106 (5·7%)

Data are mean (95% CI) or n/N (%). Except for the duration of gametocyte carriage, all estimates were adjusted for gametocyte density at enrolment. *p values are for comparison with reference 0·75 mg/kg treatment group. †Calculated for all children who had gametocytes on the day of primaquine or placebo administration.

Table 3: **Gametocyte carriage during follow-up for the different treatment regimens**

after enrolment, although 170 of 345 (49·3%) participants who were gametocyte positive at enrolment remained so on day 2 before receiving primaquine or placebo. After day 2, the rate of gametocyte clearance was dependent on treatment group. The mean duration of gametocyte carriage was 6·6 days (95% CI 5·3–7·8) in the 0·75 mg/kg reference group, 6·3 days (5·1–7·5) in the 0·4 mg/kg group, 8·0 days (6·6–9·4) in the 0·1 mg/kg group, and 12·4 days (9·9–15·0) in the placebo group (table 3). The duration of gametocyte carriage for children who were gametocyte positive at primaquine administration was the primary outcome and was tested for non-inferiority to the 0·75 mg/kg reference group. With the proposed non-inferiority margin of 2·5 days, the 0·4 mg/kg group showed non-inferiority to the reference 0·75 mg/kg group, but the 0·1 mg/kg group was inconclusive for non-inferiority and placebo was inferior (figure 2).

The mean circulation time of gametocytes indicated a longer circulation time of gametocytes in the 0·1 mg/kg group (p=0·0012) and the placebo group (p<0·0001) than

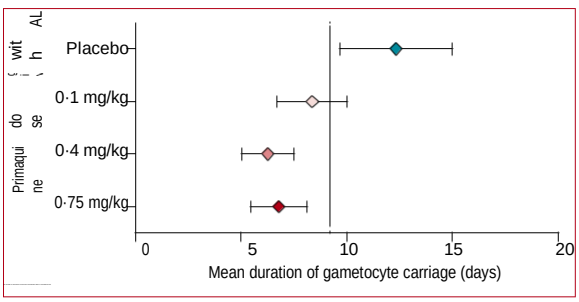

Figure 2: **Mean duration of gametocyte carriage by treatment regimen**

deterministic compartmental mathematical model to repeated *Pfs25* quantitative real-time nucleic acid sequence-based analysis gametocyte prevalence estimates. Symbols indicate the mean duration of gametocyte carriage, and error bars represent the upper and lower limit of the 95% CI. The dashed line indicates the set threshold for non-inferiority compared with the 0·75 mg/kg reference group (non-inferiority margin of 2·5 days). AL=artemether–lumefantrine.

in the reference 0·75 mg/kg group (table 3). Gametocyte circulation time did not differ significantly between the 0·4 mg/kg group and the reference 0·75 mg/kg group (p=0·80). Compared with the reference 0·75 mg/kg group, gametocyte prevalence was significantly higher in the 0·1 mg/kg group on days 7 and 10, and significantly higher in the placebo group on days 7, 10, and 14 (table 3). We recorded no difference in prevalence between the 0·4 mg/kg group and the reference group throughout follow-up (table 3, figure 3). The overall geometric mean gametocyte density was 17·9 gametocytes per µL (95% CI 13·8–23·3) at enrolment, 15·7 gametocytes per µL (11·0–22·2) on day 2 before primaquine treatment, 11·6 gametocytes per µL (7·2–18·8) on day 3, 5·3 gametocytes per µL (3·0–9·3) on day 7, 5·2 gametocytes per µL (2·6–10·5) on day 10, and 2·1 gametocytes per µL (0·7–5·7) on day 14. This decrease in the density of gametocytes in gametocyte-positive people during follow-up was statistically significant (p<0·0001) but densities in these patients did not differ significantly between treatment groups on discrete follow-up days (data not shown).

The AUC of gametocyte density over time, a measure that incorporates both prevalence and density of QT-NASBA estimates, was 3·8 (95% CI 1·7–8·2) gametocytes per µL per day in the placebo group, 3·8 (1·8–7·8) in the 0·1 mg/kg group, 2·1 (1·0–4·5) in the 0·4 mg/kg group, and 2·0 (0·9–4·3) in the 0·75 mg/kg group. After adjustment for gametocyte density at enrolment, the AUC compared with the reference group did not differ significantly for the 0·4 mg/kg group (p=0·79) or the placebo group (p=0·16), but was significantly higher in the 0·1 mg/kg group (p=0·043; data not shown). None of the efficacy estimates were affected by the sex of the participants (data not shown).

The mean maximum decrease in haemoglobin concentration did not differ significantly compared with placebo (10·7 g/L, SD 11·1) in the 0·1 mg/kg (11·4 g/L, 9·4; p=0·61), 0·4 mg/kg (11·3 g/L, 10·0; p=0·67), or

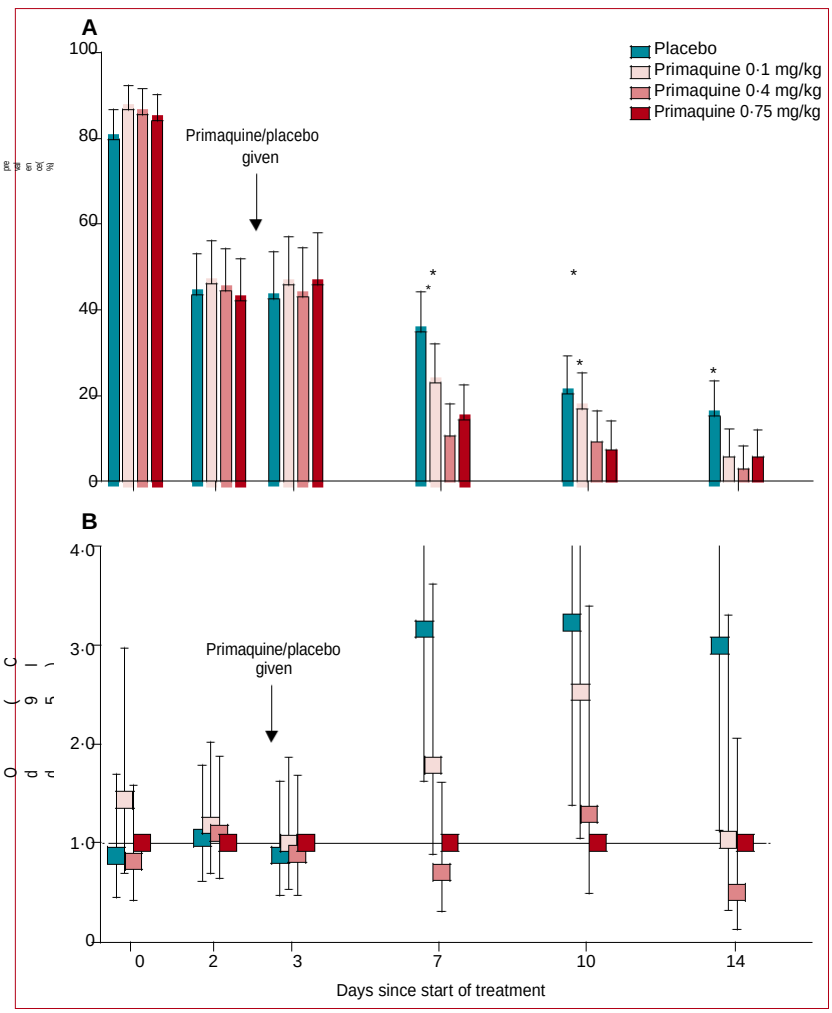

Figure 3: **Gametocyte prevalence and prevalence ratio for each treatment regimen during 14 day follow-up**
(A) Gametocyte prevalence during follow-up, as measured by *Pfs25* quantitative real-time nucleic acid sequence-based analysis. Error bars indicate the upper limit of the 95% CI. (B) Odds ratio of gametocyte prevalence on each of the days of follow-up compared with the reference 0·75 mg/kg group after adjustment for baseline gametocyte density. Error bars indicate the upper and lower limits of the 95% CI. *Indicates a statistically significant difference compared with the reference 0·75 mg/kg group.

0·75 mg/kg (12·7 g/L, 8·2; p=0·11) groups. The size of the fall in haemoglobin concentration was not significantly associated with primaquine dose (p=0·46). The timing of the nadir in haemoglobin was independent of treatment group, and the greatest contribution to the total decrease in haemoglobin occurred before day 2 when the study drug was administered. By day 28, in all treatment groups, haemoglobin concentrations had recovered and exceeded baseline concentrations (figure 4). We recorded no cases of black water fever; red, black, or tea-coloured urine; or severe haemolysis; and no child needed a blood transfusion. Sex had no effect on safety outcomes (data not shown).

The proportion of participants having adverse events did not differ between treatment groups after adjustment of significance levels for multiple comparisons (data not

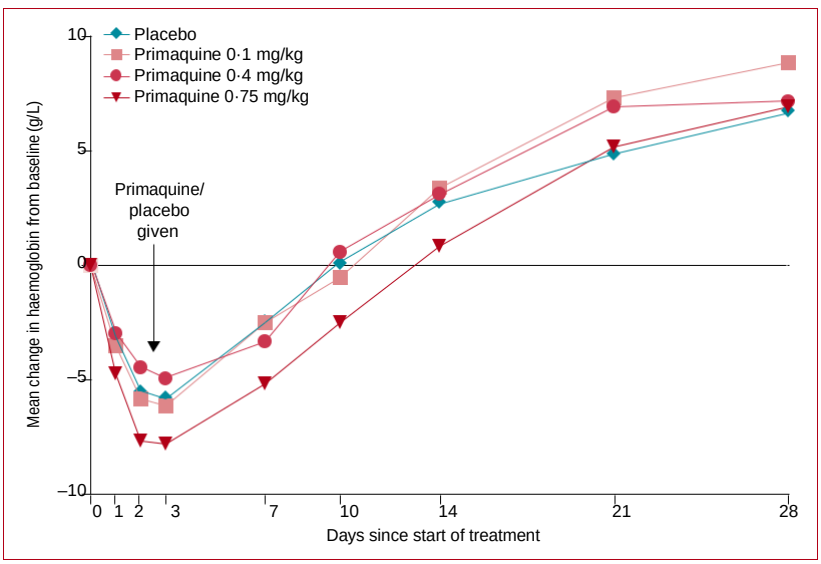

Figure 4: **Mean change in haemoglobin measurements by treatment regimen during 28 day follow-up**
Haemoglobin concentrations (g/L) during follow-up are expressed relative to that at enrolment for each treatment group.

shown). In the sex-stratified analysis, the maximum reduction in haemoglobin concentration seemed to be larger in the 0·75 mg/kg group compared with the placebo group in girls (p=0·023), but this difference was not statistically significant after correction for multiple comparisons (Bonferroni threshold level for significance p=0·0083). One child, aged 1·5 years, had a haemoglobin concentration of less than 50 g/L, which was the only severe adverse event. This boy, who received 0·4 mg/kg primaquine, had a baseline haemoglobin concentration of 99 g/L. On day 9 of follow-up, he underwent an elective surgical procedure in a mobile clinic. The mother reported no attempt at haemostasis postoperatively and the child had bled severely. By day 14, his haemoglobin concentration had fallen to 49 g/L without clinical compromise. After wound care and treatment with iron and folate, his haemoglobin concentration recovered to 106 g/L on day 28. This event was judged to be unrelated to the study drug.

Discussion

This study is the first formal dose-finding trial to assess *P falciparum* gametocyte clearance after treatment with single-dose primaquine when given in combination with an ACT (panel). We showed that the duration of gametocyte carriage was roughly halved when 0·75 mg primaquine per kg was given in addition to ACTs. A reduced dose of 0·4 mg/kg had a non-inferior gametocytocidal effect compared with the WHO reference dose, whereas the duration of gametocyte carriage was inconclusive for non-inferiority in the 0·1 mg/kg group and gametocyte prevalence was higher during follow-up than at baseline. Safety outcomes did not differ significantly between the treatment groups.

In this population of children with uncomplicated clinical malaria, gametocytes were detected at baseline in a quarter of children by microscopy compared with four-fifths by molecular methods, which is consistent with previous findings and emphasises the inadequate sensitivity of microscopy in identification of potentially infectious people.[31] Gametocyte prevalence decreased during follow-up; roughly half of the patients with gametocytes at enrolment cleared their gametocytes during the first 2 days of treatment, before primaquine was given. These dynamics differ from those reported in children in a previous ACT–primaquine trial that showed a more gradual reduction in gametocyte prevalence after ACT,[7] but are similar to those recorded in symptomatic Kenyan children of the same age group.[3] Although primaquine shortened the duration of gametocyte carriage, we noted that even the highest single dose of the drug did not render all participants gametocyte negative. In previous studies in Burma and Indonesia, microscopic gametocytes persisted in a few individuals 21 days after primaquine treatment.[8,9] In our study, six of 106 (5·7%) children were gametocyte positive by molecular methods on day 14 after initiation of treatment, even with the highest dose of primaquine. However, the density of these persistent gametocytes was much lower than that at enrolment. We used gametocyte density estimates for secondary outcome measures because no clear lower threshold gametocyte density that is needed for successful mosquito infection has been established.[32–34] The gametocyte circulation time, which was calculated on the basis of the rate of decrease of gametocyte densities after treatment, was significantly longer in the placebo and 0·1 mg/kg groups than in the reference group, but did not differ significantly between the 0·4 mg/kg group and the reference 0·75 mg/kg group. The AUC of gametocyte density over time, a summary measure for malaria transmission potential,[7,27,35] was numerically higher in the placebo group and 0·1 mg/kg dose group than in the 0·75 mg/kg dose group, but this difference was statistically significant only for the 0·1 mg/kg dose group. There was no siginficant difference in the AUC between the 0·4 mg/kg and the 0·75 mg/kg dose groups. Baseline differences in asexual parasites between treatment groups did not result in differences in baseline gametocyte prevalence or density or differences in treatment outcome, and did not confound the comparison of gametocyte dynamics between groups.

Although we used sensitive molecular gametocyte detection methods in our trial and therefore provide detail that is absent from most other primaquine trials, a relevant shortcoming of this and other studies is that gametocyte infectiousness to mosquitoes was not established. A proportion of the gametocytes that are observed by microscopy shortly after primaquine treatment might be non-infectious.[15] Whether or not *Pfs25* mRNA can be detected from non-viable gametocytes is unknown, and a proportion of the gametocytes that we detected could have

been non-infectious. We might, therefore, have underestimated the transmission-blocking effect of primaquine. None of the available gametocyte detection devices allow inferences to be made about the infectiousness of gametocytes to mosquitoes, and only mosquito feeding assays can provide definitive evidence for the transmissibility of gametocytes. However, limitations do exist in the extent to which labour-intensive mosquito feeding assays can be used in clinical trials.[36] Although gametocyte measurements can be done repeatedly from the same patient, the few clinical trials that have used mosquito feeding assays typically do feeding experiments at one timepoint per participant[3,37,38] and thereby ignore the dynamics of gametocyte infectivity.[38] Future studies that investigate the gametocytocidal effects of low-dose primaquine should therefore preferentially include mosquito feeding assays at intervals during follow-up.

A further limitation of this study was the absence of available paediatric dose formulations for primaquine, which necessitated titration of crushed primaquine in solution for accurate dosing. Although crushed tablets have been used previously for the 0·75 mg/kg dose,[7,8] this approach might have affected efficacy, especially of the lowest dose (0·1 mg/kg). More data for the relative bioavailability of different formulations of primaquine are needed. Hence, a prerequisite to the scaling up of primaquine deployment will be the availability of reliable paediatric formulations for low doses of the drug.

This study aimed to establish the efficacy and safety of low-dose primaquine in people with normal G6PD enzyme function. G6PD-deficient children were excluded from this study based on the fluorescent spot test, the most widely used enzyme function test[13] that detects enzyme function to a cutoff of about 20–30% of normal activity.[39] We decided to exclude G6PD-deficient children so that we could first establish the lowest efficacious dose before vulnerable patients are exposed to a potentially haemolytic drug. Although haemolysis has been reported in people without common mutations in the G6PD enzyme,[29] the exclusion of those with abnormal enzyme function does clearly limit the generalisability of the safety outcomes of this study and this issue needs to be addressed in future studies. Given this caveat, haemoglobin concentrations fell most rapidly in the first 2 days after enrolment in all study groups, which implies that the greatest effect on haemoglobin was caused by clinical malaria rather than a drug effect. Thereafter, haemoglobin recovered to premorbid concentrations. A similar trend has been recorded in children in Tanzania,[7] and in populations in Burma[30] and Indonesia.[9] We recorded no children with objective measures of clinically significant haemolysis or black urine, or who needed hospital admission or blood transfusion. The only severe adverse event was in a child who underwent an elective surgical procedure unrelated to the clinical malaria episode on day 9 and therefore after the expected duration of primaquine-associated haemolysis.

In this dose-finding trial, primaquine administration was delayed until day 2 after initiation of schizonticidal therapy. This timepoint is when, in the context of uncomplicated malaria, the rate of malaria-attributable haemolysis is expected to be falling, and comparisons of haematological effects between dose groups are expected to be less affected by the consequences of acute malaria infection. In operational terms, administration of primaquine on the first day of schizonticidal treatment is probably advantageous, and comparisons of the efficacy of day 0 versus day 2 administration will be important.

For more than 40 years, WHO has recommended a single dose of 0·75 mg primaquine base per kg in

Panel: Research in context

Systematic review

We searched PubMed on May 25, 2013, without date or language restrictions, with the terms "primaquine" and "malaria, falciparum" and "gametocyte" or "primaquine" and "malaria, falciparum" and "transmission". We identified no randomised controlled trials assessing the dose-response relation of primaquine for gametocytocidal activity.

A Cochrane review of the transmission-reducing efficacy of primaquine published in September, 2012, identified five trials assessing a primaquine–artemisinin combination therapy combination that satisfied the criteria for inclusion and none of these analysed a range of doses.[28] Three studies have assessed the haematological safety of primaquine with artemisinin combination therapies,[7,29,30] but our trial is unique in that it was specifically powered to assess safety outcomes. A search of clinical trial registration sites for primaquine dose-finding trials for transmission blocking showed one trial that is underway in The Gambia (NCT01838902) to assess the efficacy of artemisinin combination therapy alone, and with 0·2 mg/kg, 0·4 mg/kg, or 0·75 mg/kg primaquine base in asymptomatic patients. This trial is scheduled for completion in 2015. Another study (NCT01743820), which is in development, will assess primaquine dose escalation from 0·125 mg/kg in 50 participants randomly allocated to different dosing groups. Several other registered studies with primaquine for *Plasmodium falciparum* do not involve dose-finding but will address relevant questions for the future wide-scale deployment of primaquine. These studies include a trial of the optimum timing of primaquine administration (NCT01906788, recruiting), primaquine pharmacokinetics (NCT01552330 and NCT01525511, both completed August, 2013), and a trial with mosquito feeding as an endpoint that will compare artemisinin combination therapy alone with 0·75 mg/kg primaquine (NCT01849640, not yet recruiting, with a scheduled 3-year timeline).

Interpretation

This study is, to our knowledge, the first randomised, placebo-controlled trial to assess the dose-response relation of one dose of primaquine for gametocyte clearance and for safety in falciparum malaria. This trial was undertaken in African children with clinical malaria and normal glucose-6-phosphate dehydrogenase enzyme function. A dose reduction to 0·4 mg/kg primaquine base had demonstrable non-inferiority to the reference 0·75 mg/kg dose, whereas a dose of 0·1 mg/kg was inconclusive for non-inferiority. This trial was designed and started before a revision of the WHO guidelines recommending 0·25 mg/kg primaquine for transmission blocking, in light of which this new dose must now be assessed. In this population, all doses of primaquine had similar safety profiles to placebo. An study of low-dose primaquine in people with glucose-6-phosphate dehydrogenase deficiency is warranted.

combination with schizonticidal drugs to reduce transmission of malaria.[40] However, no dose-finding trials underpinned this recommendation. The small evidence base for primaquine use has prompted uncertainty as to the benefit of an intervention that carries a documented risk of haemolysis in malaria-endemic populations.[28,41] The real threat of spreading artemisinin resistance[42] has led to urgency in addressing this problem. In September, 2012, while our study was ongoing, an evidence review group commissioned by WHO revised its recommended dose to 0·25 mg primaquine base per kg to be added to ACT to treat parasitologically confirmed falciparum malaria infection in new programmes for malaria elimination and to stop the spread of artemisinin resistance.[43] This dose revision was based on underpowered historical studies, and the need for contemporary data was emphasised.[44] The 0·25 mg/kg dose was not assessed in our study, which is a limitation and leaves important questions to be addressed in future dose-finding trials. However, we have shown that gametocytocidal efficacy is retained when the primaquine dose is reduced from 0·75 mg/kg to 0·4 mg/kg and that a dose-response relation exists for lower doses. The finding of reduced gametocytocidal efficacy at doses lower than 0·4 mg/kg seems to contradict suggestions of uniform efficacy in the range of 0·065–0·75 mg primaquine per kg.[16] This new information provides a valuable starting point for identification of the most efficacious and safest low dose of primaquine for transmission blocking. Subsequent investigations of primaquine should include assess ments of the efficacy of doses lower than 0·4 mg/kg (including the newly recommended 0·25 mg/kg dose), with use of mosquito transmission endpoints to allow for differences in infectiousness of gametocytes persisting after treatment; the optimum timing of primaquine in combination with ACT; the pharmacokinetics of low-dose primaquine; and the safety of low-dose primaquine in people with G6PD enzyme deficiency, which is of high priority. Because of differences in gametocyte dynamics between African and Asian settings[45] and differences in the severity of G6PD deficiency across regions,[46] studies in a range of malaria-endemic settings are needed.

Contributors

ACE, TB, SY, NJW, ELW, SGS, and CD designed the study and were involved in interpretation and writing. ACE implemented and led the study. JB and ELW provided statistical support for data analysis. AO and GG participated in data collection. LG and KHWL did the QT-NASBA laboratory analysis. MK, HW, AM, and SN provided logistical support. All authors reviewed and approved the final version.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

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3.2 Reporting for non-inferiority analysis

The results were interpreted according to guidance from the Consolidated Standards of Reporting Trials (CONSORT) group guidelines for reporting non-inferiority trials (210). The 0.1mg/kg primaquine base dose outcome was analogous to scenario F in figure 4-1, below.

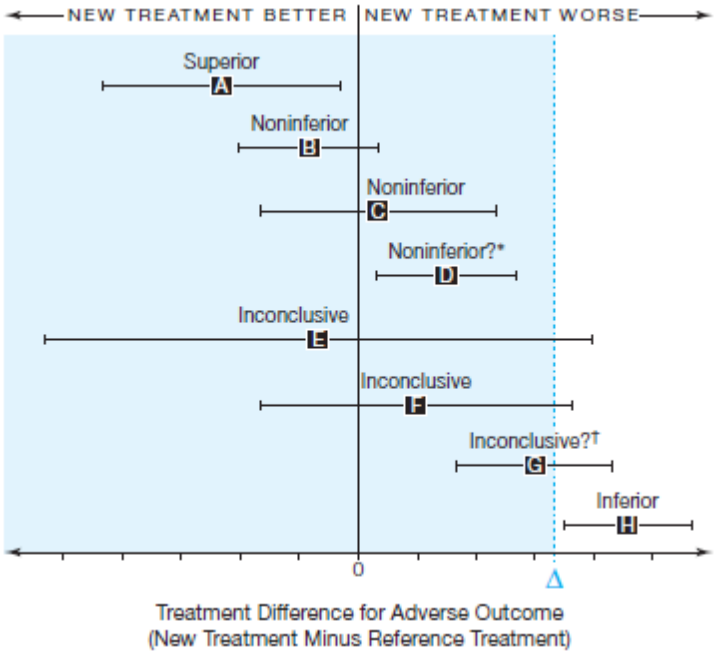

Figure 3-1 Possible scenarios of observed treatment differences for outcomes in non-inferiority trials, from Piaggio, 2012, with original figure legend (210)

Error bars indicate 2-sided 95% confidence intervals (CIs). The blue dashed line at x=Δ indicates the non-inferiority margin; the blue tinted region to the left of x=Δ indicates the zone of inferiority. A, If the CI lies wholly to the left of zero, the new treatment is superior. B and C, If the CI lies to the left ofΔ and includes zero, the new treatment is non-inferior but not shown to be superior. D, If the CI lies wholly to the left of Δ and wholly to the right of zero, the new treatment is non-inferior in the sense already defined but also inferior in the sense that a null treatment difference is excluded. This puzzling circumstance is rare, because it requires a very large sample size. It also can result from a non-inferiority margin that is too wide. E and F, If the CI includes Δ and zero, the difference is non-significant but the result regarding non-inferiority is inconclusive. G, If the CI includes Δ and is wholly to the right of zero,

the difference is statistically significant but the result is inconclusive regarding possible inferiority of magnitude Δ or worse. H, If the CI is wholly above Δ, the new treatment is inferior.

3.3 Additional results and considerations not included in peer reviewed publication

3.3.1 Interpolation to incorporate a 0.25mg/kg dose arm

Upon study completion, the WHO revised recommendations for single dose primaquine, proposing a lower single dose of 0.25mg/kg primaquine base for malaria transmission-blocking (257). Since this dose was not included in the trial arms, a short exercise was undertaken to use visual interpolation to predict the outcome a notional 0.25mg/kg dose arm for this trial (Figure 3-2) and to present the thesis trial results in the context of two studies that used an identical method to assess primaquine efficacy as used in this thesis, one of which incorporated the new WHO 0.25mg/kg dose as a trial arm (Figure 3-3).

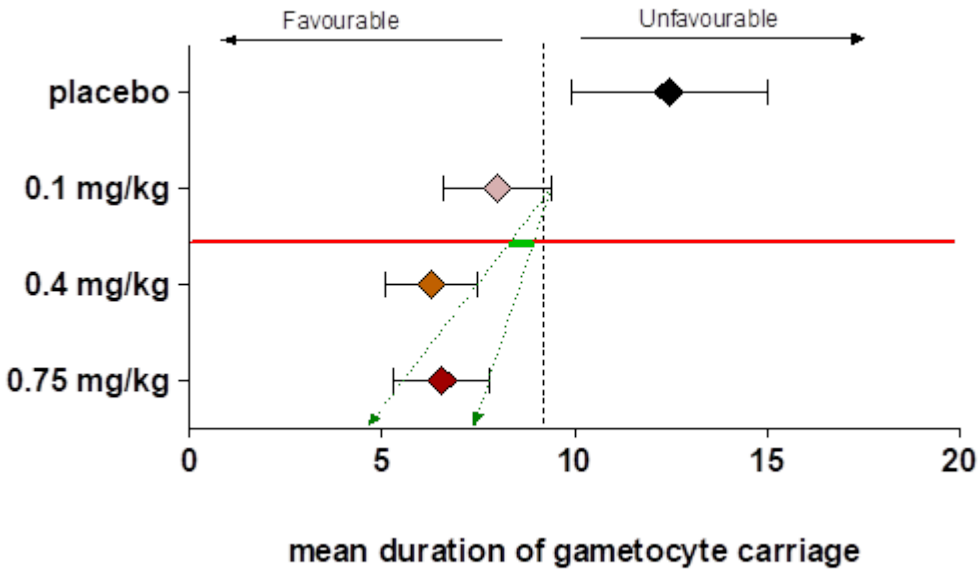

Figure 3-2 Non-inferiority analysis of the number of days to gametocyte clearance for each primaquine dose arm. Interpolation to predict the outcome of a notional 0.25mg/kg primaquine dose arm for the trial

Visual interpolation was used to predict the range of values for the upper 95% confidence limit of a notional 0.25mg/kg primaquine dose arm for the thesis trial. The red line denotes the primaquine dose of 0.25mg/kg. The green line highlights the range of values predicted for the upper 95% confidence limit for the 0.25mg/kg dose arm. The black dotted line represents the non-inferiority margin used for the thesis trial. Given that the upper 95% confidence limit for the interpolated 0.25mg/kg dose outcome does not cross the non-inferiority margin, it would be interpreted as having non-inferior efficacy to the WHO reference dose of 0.75mg/kg primaquine base.

The limited number of dose arms (n=4) in the thesis trial reduced the statistical validity of inferring the outcome of postulated intermediate dose arms. With this in mind, a simple interpolation was undertaken to predict where a 0.25mg/kg dose arm (the revised WHO-recommended single dose of primaquine) would fall with respect to the non-inferiority margin used for this trial. Figure 3-3 represents a prediction of the upper 95% confidence limit for an interpolated 0.25mg/kg dose arm. It was based on an assumption of linearity that this value

would fall between the upper 95% confidence limit of the 0.1mg/kg dose arm and either of the two higher dose arms. This highly simplistic interpolation suggests that the upper 95% confidence limit for the 025mg/kg primaquine dose arm would be expected to fall on the favourable side of the non-inferiority margin, conferring non-inferior efficacy to the reference 0.75mg/kg dose arm. It does, however, lie close to the non-inferiority margin, pointing to the importance of well-designed studies incorporating the 0.25mg/kg primaquine dose arm in order to determine its efficacy.

Data from a Tanzanian trial of ACT alone and ACT with 0.75mg/kg primaquine (258) and from the first sister study to this trial that included the 0.25mg/kg dose arm, (conducted in Burkina Faso) (259) were incorporated into the trial results presentation Figure 3-3. The non-inferiority margin was consistent with that used in this trial (256). Doses of 0.25mg/kg and above were non-inferior to the 0.75mg/kg dose for gametocyte clearance.

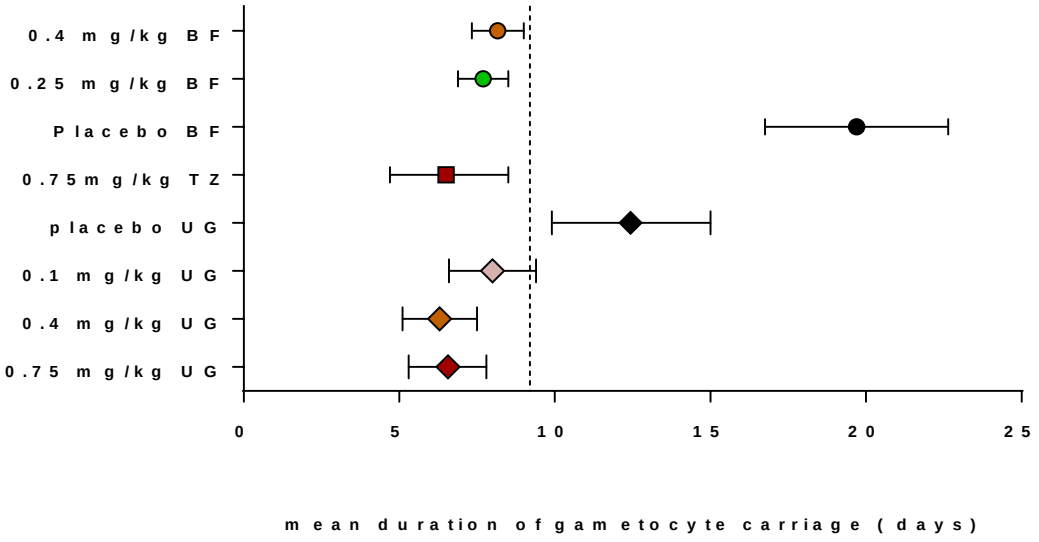

Figure 3-3 Mean duration of gametocyte carriage, in days, by treatment given for three trials of artemisinin combination therapy (ACT) with and without primaquine for gametocyte clearance, in Tanzania (TZ), Burkina Faso (BF) and Uganda (UG).

The data from clinical trials in Tanzania (258) and Burkina Faso (259), that used identical methods to assess primaquine's gametocyte clearance efficacy in children with uncomplicated malaria, are presented alongside data from this trial in Uganda (256). Dose arms from each study are plotted separately. Each colour represents a specific dose. The non-inferiority margin of 2.5 days from the Ugandan reference dose arm is marked (dotted line). Non-inferiority to the 0.75mg/kg dose is found for doses of 0.25mg/kg and above.

3.3.2 The effect of symptomatic malaria infection on mean maximal fall in haemoglobin during follow up

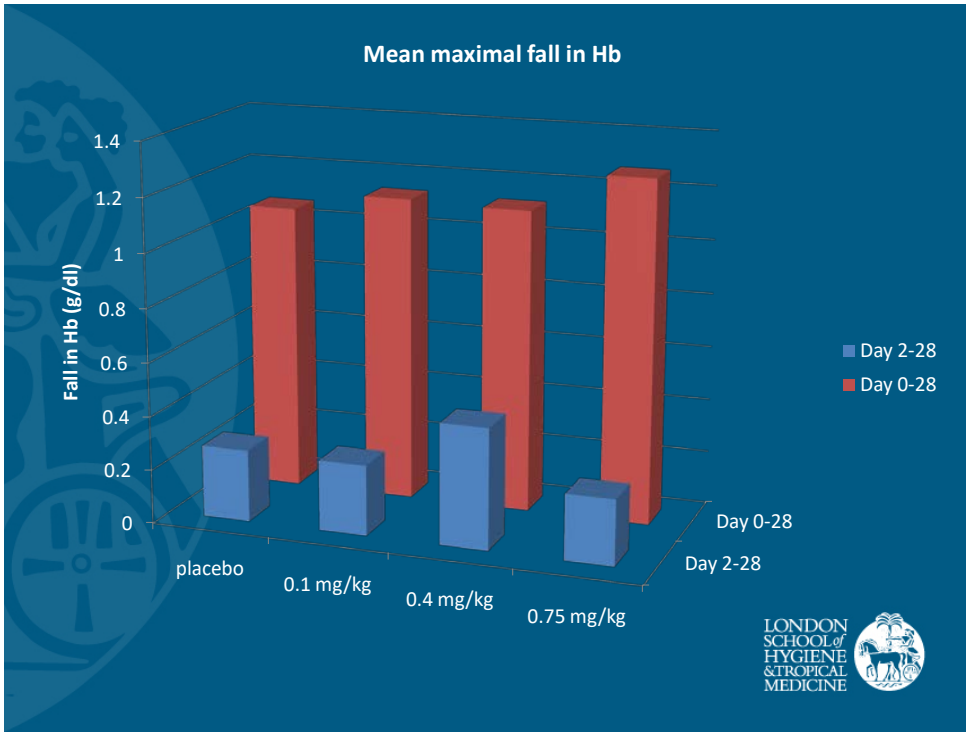

Figure 3-4 Comparison of the mean maximal fall in haemoglobin over 28 days of follow up with and without the inclusion of day 0 and day 1 haemoglobin measurement

Bars represent the mean maximal fall in haemoglobin per primaquine dose treatment group from enrolment to the end of follow up (day 0-28: red bars), and from administration of primaquine to the

end of follow up (day 2-28; blue bars). The greatest fall in haemoglobin occurs in the period from day 0 to day 2 for these children, i.e., prior to primaquine treatment and during artemether-lumefantrine treatment for symptomatic malaria infection.

Primaquine was administered on day 2, after blood had been drawn for day 2 analysis. The largest fall in haemoglobin happened between day 0 and day 2; for all treatment arms, the fall in haemoglobin that occurred between days 0 to 2 was more than twice the size of the fall between day 2 and the end of follow up on day 28 (Figure 3-4). Hence, the greatest haemoglobin fall is during the period of acute clinical malaria infection, where haemolysis might be attributed to either malaria or, possibly artemether-lumefantrine treatment, but not to primaquine treatment.

3.3.3 Safety events: Consultations with regional lead paediatrician

Two study participants had medical conditions during the trial that prompted the need for review by a consultant paediatrician at the Regional Paediatric Referral Hospital in Jinja. Details of both cases were presented to the DSMB for review. The two cases are outlined below.

Case 1:

A 2-year-old boy developed fixed rotation of his neck, resembling a torticollis, on day 11 of participation. Passive movement of his head did not appear painful and he was otherwise well, afebrile and continued to play with toys with his siblings. The consultant paediatrician held the opinion that this was musculoskeletal in origin. Simple analgesia was provided and the child's neuromuscular signs resolved after three days. The DSMB was consulted and they decided that this did not represent a severe adverse event, nor should it be reported as related to the study drug. It was recorded as an adverse event.

Case 2:

A 5-year-old boy was found to have a significant fall in haemoglobin count on day 14 of recruitment. From a baseline of 9.9 g/dL at enrolment, his haemoglobin fell to 6.8 g/dL on day 10 and to 4.9 g/dL on day 14. On questioning, his mother revealed that she had taken her son to a private clinic where he was circumcised on day 9 after enrolment into the study. No attempt had been made to maintain haemostasis following the circumcision procedure and he had bled significantly. At review on day 10, the child was in pain and mildly tachycardic. Study staff gave wound care and administered analgesia. The child was brought to the regional paediatric referral hospital for consultation, where it was confirmed that haemostasis had been achieved, he was clinically stable and no further surgical input was required. There was no systemic compromise, so it was advised that blood transfusion was not indicated, but haematinic medications were prescribed. He was monitored closely and his haemoglobin recovered to 10.6 g/dL on day 28 of recruitment. This event was reported to the DSMB contemporaneously and it was considered to be a severe adverse event but there was agreement that it was unrelated to the study drug. After un-blinding, he was found to be in the 0.4mg/kg study arm. This may explain the anomalously higher fall in haemoglobin in the 0.4mg/kg study arm after day 2 (Figure 3-4, Section 3.3.2).

3.3.4 Trial outcomes for policy development

3.3.4.1 *Development of a Single Low-dose Primaquine Working Group*

After this trial was ethically approved and had started recruiting, the first in a series of scientific meetings was held on 5th to 6th March 2012 in London, with the support of the Bill and Melinda Gates Foundation, co-hosted by the Malaria Centre at the London School of Hygiene and Tropical Medicine and the Global Health Group at the University of California,

San Francisco. This meeting led to the development of a Single Low-Dose Primaquine Working Group. The objectives of the group were "to review and discuss existing data on the use of primaquine in Africa for transmission-blocking with the aim of identifying the road blocks to its use and the necessary studies to overcome these road blocks for a wider deployment of primaquine and other transmission-blocking drugs" (250). The first meeting provided an opportunity to present the trial design for this study and to participate actively in the delineation of international research priorities to provide an evidence base for the deployment of primaquine to block malaria transmission. This trial would, therefore, become the first of a cohort of new trials addressing the identified deficiency of data to inform policy makers and the programmatic use of low-dose primaquine (260). The voice of policy makers was prominent within the contributors to the group. In addition to research investigators, in attendance were national malaria control programme directors, industry representatives and non-governmental organisation representatives. Subsequently, this group met annually to biannually, until 2016, promoting data sharing, collaboration and development of a new body of research designed prospectively to address pertinent questions. The focus of the group evolved over four years from asking efficacy and safety-based research questions in 2012 to asking drug- and intervention delivery questions in 2016. The proceedings of the first meeting were accepted for publication in a peer-reviewed journal (Section 2.3.1) (199).

3.3.4.2 *Data sharing to inform policy and further research agenda*

During the course of this research, the potential importance of primaquine as a transmission-blocker was recognised by the WHO Malaria Policy Advisory Group, prompting an Evidence Review Group to assess the safety and effectiveness of single dose primaquine as a *Plasmodium falciparum* gametocytocide in August 2012 (257). It was agreed with the trial

advisory group that the provisional trial report should be released to the WHO Evidence Review Group as pre-meeting material (Appendix C, part 2).

In the same year, PATH (an international non-profit global health organisation) commissioned an advisory workshop “to identify key technical, operational and regulatory bottlenecks for the adoption of current and emerging G6PD deficiency diagnostic tests in support of treatment of malaria and malaria elimination efforts with 8-aminoquinoline drugs such as primaquine and tafenoquine.” This trial was presented at the meeting in an advisory capacity (185).

In 2014, the Single Low-Dose Primaquine Efficacy and Safety study groups were established by the Worldwide Antimalarial Resistance Network (WWARN), providing a platform for data data-sharing from this study to enable a pooled analysis (198). Results of the pooled analysis have been presented at the European Congress on Tropical Medicine and International Health (261) and are in preparation for publication.

The trial data was also requested by and shared with the Quantitative Sciences group at the Bill and Melinda Gates Foundation in 2014, to further transparency, mathematical modelling and hypothesis building for malaria elimination.

4 Results: G6PD data analysis

4.1 RESEARCH PAPER 4: Publication of trial G6PD analysis

The analysis of G6PD data from this thesis was published in the Archives of Antimicrobial Chemotherapy (262). In this first trial of single-dose primaquine for transmission-blocking in Uganda, this paper assesses the safety of reducing doses of primaquine according to the range of G6PD genotypes in the children who were enrolled.

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RESEARCH PAPER COVER SHEET

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Student ID Number	257918/RITD	**Title**	Dr
First Name(s)	Alice Chijioke		
Surname/Family Name	Eziefula		
Thesis Title	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Antimicrobial Agents and Chemotherapy		
When was the work published?	9th June 2014		
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SECTION E

Student Signature	Chi Eziefula [redacted]
Date	18th September 2019

Supervisor Signature	
Date	23rd September 2019

Glucose-6-Phosphate Dehydrogenase Status and Risk of Hemolysis in Plasmodium falciparum-Infected African Children Receiving Single-Dose Primaquine

Alice C. Eziefula, Helmi Pett, Lynn Grignard, Salome Opus, Moses Kiggundu, Moses R. Kamya, Shunmay Yeung, Sarah G. Staedke, Teun Bousema and Chris Drakeley

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Glucose-6-Phosphate Dehydrogenase Status and Risk of Hemolysis in *Plasmodium falciparum*-Infected African Children Receiving Single-Dose Primaquine

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Glucose-6-phosphate dehydrogenase (G6PD) enzyme function and genotype were determined in Ugandan children with uncom-plicated falciparum malaria enrolled in a primaquine trial after exclusion of severe G6PD deficiency by fluorescent spot test. G6PD A heterozygotes and hemizygotes/homozygotes experienced dose-dependent lower hemoglobin concentrations after treatment. No severe anemia was observed.

Declines in malaria due to *Plasmodium falciparum* have been documented in a number of settings where malaria is endemic. It is debated whether scaling-up of conventional malaria control will sustain these declines or achieve elimination unless augmented by tools that specifically reduce transmission. Primaquine is the only currently available drug that actively clears mature *P. falciparum* gametocytes and prevents malaria transmission to mosquitoes (1). The wide-scale use of primaquine is hampered by its hemolytic effect in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency. The mutation deficiency alters G6PD enzyme function (2), exposing red blood cells to oxidative stress and resultant hemolysis in the presence of a stressor, such as primaquine (3, 4). Primaquine-induced hemolysis is dose related (1, 5, 6). While testing for G6PD deficiency is widely recommended prior to the radical treatment of *Plasmodium vivax* with 14 days of primaquine, *P. falciparum* transmission may be considerably reduced by a single, low dose of primaquine (1, 7) and may avoid the necessity to screen for G6PD deficiency. We determined G6PD enzyme function and the presence of the most common African G6PD mutation (G6PD A ; 202A/376G) in a cohort of Ugandan children treated with low-dose primaquine for clearing *P. falciparum* gametocytes. This was a randomized, double-blinded placebo controlled trial with four parallel arms. Ugandan children 1 to 10 years old with uncomplicated *P. falciparum* malaria, hemoglobin concentration (Hb) of $8 g/dl, and normal G6PD enzyme function based on a fluorescent spot test (FST; R&D Diagnostics, Agia Paraskevi, Greece) were enrolled and randomized to treatment with artemether lumefantrine (AL) alone or with a single dose of primaquine at 0.1, 0.4, or 0.75 mg/kg of body weight on the last day of AL treatment (7, 8). Genotyping of G6PD 202A and G6PD 376G was performed (9, 10). Hb was measured on days 0, 1, 2, 3, 7, 10, 14, 21, and 28 after enrollment by HemoCue 201 (Angelholm, Sweden) and expressed as absolute and relative change compared to baseline values. These values were normally distributed, presented using mean values and standard deviations, and analyzed using linear regression models. Because the age distribution of the red blood cell population influences the severity of drug-induced hemolysis (11), we adjusted all

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TABLE 1 Baseline characteristics

Characteristic	Value by G6PD 202 A genotype				
	Wild type	Heterozygous	*P* value for difference from wild type	Homozygous/hemizygous	*P* value for difference from wild type
No. of participants (% study population)	373 (80.9)	61 (13.2)		27 (5.9)	
% female (no. of females/total no. of participants)	46.7 (174/373)	100.0 (61/61)	0.001	3.7 (1/27)	0.001
Mean (SD) age in yrs	5.0 (2.6)	4.8 (2.3)	0.61	4.9 (2.4)	0.86
Mean (SD) baseline Hb concn in g/dl	11.2 (1.5)	11.4 (1.4)	0.20	10.9 (1.4)	0.38
% 376G genotype (no. of participants with genotype/total no.)					
Heterozygous	18.6 (69/371)	78.7 (48/61)		0.0 (0/27)	
Homozygous	12.9 (48/371)	21.3 (13/61)	0.001	100.0 (27/27)	0.001

TABLE 2 G6PD 202 A genotype and hemoglobin levels[a]

Characteristic	Value by treatment arm			
	0.75 mg/kg primaquine	0.4 mg/kg primaquine	0.1 mg/kg primaquine	Placebo
No. of study participants				
G6PD normal	98	90	93	92
G6PD heterozygous	14	13	16	18
G6PD hemizygous/homozygous	4	10	6	7
Mean absolute change (SD) in Hb on day 7				
G6PD normal, in g/dl	0.41 (0.95)	0.25 (1.22)	0.30 (1.07)	0.11 (1.33)
G6PD heterozygous, in g/dl	1.08 (1.14)	0.99 (1.48)	0.07 (0.98)	0.49 (1.40)
P value	0.048	0.054	0.35	0.28
G6PD hemizygous/homozygous, in g/liter	1.10 (1.34)	0.48 (0.76)	0.07 (1.21)	1.02 (0.81)
P value	0.21	0.043	0.91	0.22
% relative change (SD) in Hb on day 7, in g/dl				
G6PD normal	3.25 (8.60)	1.28 (11.24)	2.16 (9.71)	0.23 (11.34)
G6PD heterozygous	9.38 (10.4)	7.79 (12.57)	0.01 (8.56)	3.26 (12.26)
P value	0.044	0.073	0.34	0.33
G6PD hemizygous/homozygous	7.97 (12.40)	4.29 (7.70)	0.05 (11.45)	8.59 (7.28)
P value	0.36	0.028	0.93	0.16

[a] On day 7 after initiation of treatment with artemether-lumefantrine (AL) plus placebo or AL plus different doses of primaquine. All primaquine or placebo treatment was administered together with six doses of AL; primaquine/placebo was given on day 2 of treatment, together with dose 5 of AL. *P* values are compared to G6PD-normal individuals, adjusted for baseline Hb concentration.

comparisons for baseline Hb concentration. All trial participants (*n* 468) were G6PD normal by FST. DNA was available for 461 individuals of whom 27 (5.9%) were homozygous/hemizygous, 61 were heterozygous (13.2%), and 373 (80.9%) were normal for the G6PD variant A (wild type [WT]). All individuals with the 202A mutation also had the 376G mutation, and individuals were classified based on the 202A mutation (Table 1). G6PD 202 A heterozygous individuals experienced a mean reduction in Hb concentration on day 7 after treatment of 1.08 g/dl (standard de-viation [SD], 1.14; *P* 0.048) in the 0.75-mg/kg treatment arm and 0.99 g/dl (SD, 1.48; *P* 0.054) in the 0.4-mg/kg treatment arm (Table 2). Homozygous/hemizygous individuals in the 0.75-mg/kg and 0.4-mg/kg arms also experienced a reduction in abso-lute Hb concentration on day 7, although this was statistically significant in the 0.4-mg/kg arm only (*P* 0.043). When changes in Hb concentration on day 7 were expressed as a proportion of baseline Hb concentration, the same trend was observed with sta-tistically significant decreases in the 0.75-mg/kg arm for heterozy-gous individuals and in the 0.4-mg/kg arm for homozygous/hem-izygous individuals. No statistically significant changes in absolute or relative Hb concentrations were observed for heterozygous or homozygous/hemizygous individuals in the 0.1-mg/kg arm or placebo arm (Table 2). We found no explanation for the numer-ically large, but statistically nonsignificant, reduction in Hb con-centration in homozygous/hemizygous individuals on day 7 after receiving AL without primaquine. A previous study found no he-molysis after AL in homozygous/hemizygous individuals (12), and we conclude our observation may be a spurious finding and related to our small sample size. We observed no statistically sig-nificant associations between G6PD genotype and absolute or rel-ative Hb concentrations in any treatment arm on days 3 and 10 after initiation of treatment (see the supplemental material). Six-ty-nine individuals experienced a reduction of 2 g/dl in the first 2 weeks of follow-up: 13.7% (51/373) of the WT individuals, 26.2% (16/61; *P* 0.031) of the heterozygous individuals, and 7.4% (2/27; *P* 0.48) of the G6PD 202 A homozygous/hemizygous individuals (*P* 0.020). For all individuals, Hb concentra-tions normalized during follow-up. The current findings provide important data on the hemolytic effect of single-, low-dose prim-aquine. Our results show that the predominant test for G6PD deficiency screening, the FST (13), failed to identify a substantial proportion of individuals who were genotypically G6PD deficient, particularly female heterozygotes, who experienced significant re-ductions in hemoglobin following higher doses of primaquine. The observation that some G6PD-deficient individuals were FST normal is unsurprising since the test may be insufficiently sensi-tive to detect mild G6PD deficiency (13), but there are few supportive published data. We observed statistically significant decreases in Hb following single-dose primaquine in these G6PD-deficient individuals. A hemolytic effect of a single dose of 0.75 mg/kg primaquine base has been reported before (6); our study shows that a reduction in Hb concentrations is also evident after a single dose of 0.4 mg/kg but not 0.1 mg/kg. Moreover, reductions in Hb were transient, with no participant experiencing clinical symptoms suggestive of anemia and none requiring related clini-cal care. Although these findings are notable, a major limitation of the study is that individuals who were determined G6PD deficient based on the FST were excluded from the study (*n* 32), thereby plausibly removing those most severely deficient and thereby those with the highest risk of primaquine-induced hemolysis. There is therefore a need for confirmatory trials to formally assess primaquine safety in G6PD-deficient individuals, in particular with the World Health Organization recommended dose of 0.25 mg/kg. Such studies will have to take into account interindividual differences in primaquine metabolism that determine primaquine efficacy in *P. vivax* (14) and potentially also safety.

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4.2 RESEAERCH PAPER 5: Field testing for G6PD deficiency

The thesis provided the opportunity to test novel methods for assessing G6PD status in a large field-based sample. The evaluation of a high-throughput method, the WST8/1-methoxy-PMS enzymatic assay was peer-reviewed and published and is presented here (171).

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Student ID Number	257918/RITD	**Title**	Dr
First Name(s)	Alice Chijioke		
Surname/Family Name	Eziefula		
Thesis Title	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda		
Primary Supervisor	Chris Drakeley		

If the Research Paper has previously been published please complete Section B, if not please move to Section C.

SECTION B – Paper already published

Where was the work published?	Malaria Journal		
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SECTION E

Student Signature	Chi Eziefula [redacted]
Date	18th September 2019

Supervisor Signature	
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Tools for mass screening of G6PD deficiency: validation of the WST8/1-methoxy-PMS enzymatic assay in Uganda

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Tools for mass screening of G6PD deficiency: validation of the WST8/1-methoxy-PMS enzymatic assay in Uganda

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Abstract

Background

The distribution of the enzymopathy glucose-6-phosphate dehydrogenase (G6PD) deficiency is linked to areas of high malaria endemicity due to its association with protection from disease. G6PD deficiency is also identified as the cause of severe haemolysis following administration of the anti-malarial drug primaquine and further use of this drug will likely require identification of G6PD deficiency on a population level. Current conventional methods for G6PD screening have various disadvantages for field use.

Methods

The WST8/1-methoxy PMS method, recently adapted for field use, was validated using a gold standard enzymatic assay (R&D Diagnostics Ltd ®) in a study involving 235 children under five years of age, who were recruited by random selection from a cohort study in Tororo, Uganda. Blood spots were collected by finger-prick onto filter paper at routine visits, and G6PD activity was determined by both tests. Performance of the WST8/1-methoxy PMS test under various temperature, light, and storage conditions was evaluated.

Results

The WST8/1-methoxy PMS assay was found to have 72% sensitivity and 98% specificity when compared to the commercial enzymatic assay and the AUC was 0.904, suggesting good agreement. Misclassifications were at borderline values of G6PD activity between mild and normal levels, or related to outlier haemoglobin values (<8.0gHb/dl or >14gHb/dl) associated with ongoing anaemia or recent haemolytic crises. Although severe G6PD deficiency was not found in the area, the test enabled identification of low G6PD activity. The assay was found to be highly robust for field use; showing less light sensitivity, good performance over a wide temperature range, and good capacity for medium-to-long term storage.

Conclusions

The WST8/1-methoxy PMS assay was comparable to the currently used standard enzymatic test, and offers advantages in terms of cost, storage, portability and use in resource-limited settings. Such features make this test a potential key tool for deployment in the field for point of care assessment prior to primaquine administration in malaria-endemic areas. As with other G6PD tests, outlier haemoglobin levels may confound G6PD level estimation.

Keywords

Malaria, G6PD deficiency, WST8/1-methoxy PMS, Primaquine

Background

Malaria has exerted the greatest genetic pressure on the human genome in recent times, resulting in the evolutionary selection of genetic mutations that confer protection against the

disease [1-4]. Glucose-6-phosphate dehydrogenase (G6PD) is an X-linked recessive hereditary disorder that currently affects 200–400 million people worldw ide, with over 160 mutations identified [3-6] and there is pronounced geographical overlap between areas of G6PD deficiency prevalence and malaria endemicity [2,7-15]. The G6PD gene codes for an enzyme responsible for catalyzing nicotidamine adenine dinucleotide phosphate (NADP+) to its reduced form, NADPH, in the pentose phosphate pathway. Among G6PD variants with reduced enzyme activity, several phenotypic effects have been described, and are classified by the WHO as: enzyme deficiency with chronic non-spherocytic anaemia (class I, <10% activity); severe enzyme deficiency (class II, <10% activity); moderate/mild enzyme deficiency (class III, 10-60% activity); very mild or no enzyme deficiency (class IV, >60-100% activity); and increased enzyme activity (class V, >150% activity) [16]. Erythrocytes with insufficient G6PD are thus unprotected against oxidative injury, and individuals with G6PD deficiency may develop haemolytic anaemia in response to a number of stresses, including infection and exposure to medications such as the 8 amino-quinoline, primaquine [17].

Primaquine has received renewed interest in the context of malaria eradication. The drug is recommended as presumptive anti-relapse treatment of *Plasmodium vivax* and *Plasmodium ovale* infection due to its activity against hypnozoites. Furthermore, it remains the only readily-available drug that actively clears mature *P. falciparum* gametocytes [18-22]. Given the risk of haemolysis in G6PD deficient individuals, and the genetic and phenotypic variability of G6PD deficiency across geographic areas where primaquine treatment is considered, estimation of G6PD enzyme function prior to drug administration is recommended [23]. At present, however, primaquine therapy without prior determination of G6PD enzyme function, perhaps due to a lack of reliable tests, is thought to be common [24].

One possible reason for the current lack of a standard diagnostic test is that the majority of methods for assessing G6PD deficiency have shortcomings for field use in tropical countries [25-27] (see Table 1). In 2003, a novel enzymatic method to detect G6PD deficiency was developed [28], based on the WST8 tetrazolium salt and the 1-methoxy PMS hydrogen carrier. The assay has reduced light sensitivity, and is easily interpretable, both quantitatively and qualitatively. In 2010 Kuwahata *et al.* reported a version of this method, optimized for use in a 96-well plate format using dried bloodspots in filter paper, which was successfully tested as an in-field mass-screening tool for G6PD deficiency in the Solomon Islands [25]. The aims of this current study were to further validate the WST8/1-methoxy-PMS test by comparison with a commercially available enzymatic reference test and to assess the test's robustness for field use.

Table 1 Available tests for determination of G6PD deficiency and their use in field settings

Test	Characteristics	Shortcomings for field and mass-screening
DNA sequence analysis of the G6PD gene.	Extremely reliable. Primers are used to check whether the G6PD gene contains a mutation.	Requires training, and equipment. Genotype does not correlate with enzyme function and the risk of haemolysis. Female heterozygous have unpredictable phenotype due to X chromosome lyonization. Only one mutation can be analysed with one primer (>160 mutations exist).
Brilliant cresyl blue decolouration test	Involves the action of G6PD and NADPH diaphorase. A deficiency of either one of these enzymes on RBCs would result in the brilliant cresyl blue remaining unchanged in the test.	Laborious processes; requires technical skill, and has low sensitivity.
Methaemoglobin reduction test	Based on the oxidation of Hb to MetHb by sodium nitrate and the subsequent enzymatic reconversion to Hb in the presence of methylene blue.	Laborious, qualitative and low sensitivity. Does not enable identification of heterozygous deficient females.
Formazan ring method	Uses the principle of the MTT-Linked spot test. When G6PD is present at normal levels, MTT is reduced to a purple insoluble formazan derivative, and results in a specific diameter of discolouration.	Prone to misdiagnosis.Ring thickness may be affected by exogenous factors.
Sephadex gel MTT-PMS method	Mostly used in Asia, and predecessor in concept, of the WST8/1-methoxy PMS test.	Reacts with haemoglobin; is light sensitive and water insoluble. It is of a qualitative nature.
Fluorescent spot test (FST)	ICSH-recommended method.	Its cut-off value for G6PD deficiency determination is only 10-20% of the normal G6PD activity, which excludes patients with moderate enzyme deficiency and increases the risk of false-normal diagnosis.
BinaxNOW® rapid test	Rapid test format: Overcomes i ssues of technical skill, sophisticated equipment and reliability.	It is highly dependent on temperature-sensitive kinetic enzymatic reactions. This limits its use to areas with temperatures between 18 and 25C. Potential cost.
CareStart ^{TM} test	RDT format. Qualitative chromatographic test, based in the reduction of colourless nitro blue tetrazolium dye to dark colour formazan. Long-term temperature stability.	Potential cost.
R&D® enzymatic test (reference)	Both depend on the conversion of NADP + to NADPH by G6PD.	Enzymatic gold standard. Requires various temperature-dependent incubations.
WST8/1-methoxy PMS test (test under validation)	NADPH converts colourless tetrazolium salt into a coloured formazan, while NADP + does not.	Evaluated in this work. Advantages: no reaction with haemoglobin, lower light sensitivity.

Methods

Study site & sample selection

The study was conducted in seven sub-counties (Nagongera, Paya, Kirewa, Kisoko, Petta, Mulanda, and Rubongi) in Tororo district, an area with very high malaria transmission intensity in Uganda. In August-September 2010, the study area was mapped and a census

survey carried out. Households within 2km of a health facility were included in the sampling frame. Children under the age of five years were recruited from randomly selected households and were enrolled into a cohort study (Clinical Trials registration number NCT01024426) if they met the following inclusion criteria: 1) age < 5 years, 2) agreement of parents or guardians to provide informed consent, 3) no intention to move during the follow-up period. Clinical and laboratory evaluations were conducted at enrolment and repeated every six months over the period of follow-up. Blood samples collected from cohort study participants at follow-up visits conducted in July and August 2011 were used for the G6PD study.

Laboratory procedures

Blood samples were collected by finger-prick, onto 3MM filter paper, and were dried at ambient temperature. Samples were then stored at room temperature in zip-lock bags containing silica desiccant beads, and assayed within 24-72h. The remainder of the sample was stored for various time periods and temperature/illumination conditions for further evaluation. Additionally, haemoglobin values were obtained using a HaemoCue B analyser. In parallel, two sets of internal controls were generated to calibrate the assay. A commercial standard reagent of known G6PD activity (Trinity Biotech Normal Control) was used to create a panel of normal, moderate, and severe deficiency (100%, 30% and 10% activity respectively), as well as a no-enzyme control (0%). The second set of internal controls was generated from human blood from two volunteers with normal G6PD activity, and followed the procedure described for the field-adapted test [25]. Each set of controls were spotted onto 3MM filter paper (Whatman), and stored under the same conditions as the samples. Blood spots and controls were tested by both the optimized WST8/1-methoxy PMS assay, and by the commercially available standard R&D® test. Results were evaluated both visually and quantitatively using a spectrophotemer at 450 nm.

WST8/1-methoxy PMS assay

The principle of the WST8/1-methoxy PMS method depends on reducing hydrogen from NADPH converting WST8 to WST8-formazan in the presence of the hydrogen carrier 1-methoxy-PMS. This reaction yields a strong easily detectable orange colour, with colour intensity directly proportional to G6PD activity. After a 2hr incubation at room temperature, samples with normal G6PD activity show strong orange colour, deficient samples show faint colour (moderate deficiency likely to represent heterozygotes) or no colour (severe deficiency & negative controls).

Two stock solutions were prepared: a working mix, and a control mix. The working mix contained 50mM G6P (Roche), 4mM NADP (Merck Pty Ltd), 1M Tris–HCl pH 7.2-7.5, and 100mM MgCl2 (Sigma-Aldrich). The control mix contained all reagents in the concentrations described above, but lacked NADP and G6P. Mixes for assay development consisted of 0.5mL of WST8/1-methoxy PMS (Dojindo Laboratories), 0.5mL of working stock solution, and 19mL of distilled water for every 96-well plate. Negative controls were generated on site as described in previous studies [25].

A 1.5mm diameter disc was punched out from each blood spot sample and placed inside a single well of the 96-well flat bottom microplate. Samples were assessed in duplicate. Plates were incubated for 2h at ambient temperature, and were then inspected by eye by two different observers for qualitative analysis. For quantitative analysis, the optical density was

quantified in a microplate reader (Multiskan EX, Thermo scientific) at wavelength OD450-594nm. G6PD levels were determined in reference to the control panels.

Reference assay: standard quantitative G6PD assay (R&D® diagnostics)

The R&D® colourimetric test was used for validation [29,30]. In this t est, the resulting NADPH reacts with a colour reagent in which a formazan salt (nitrotetrazolium blue) is produced, generating a visually detectable purple colour. The resulting OD (measured at 550nm), is proportional to the level of G6PD present in the dried sample. The assay was performed using 96-well plates and dried blood-spots in filter paper as per manufacturers instructions. The same sets of controls were used for both assays, and their robustness tested under various temperature, storage and light conditions.

Experiments to assess assay robustness

Storage

Storage of 150 dried blood spots (FPBS) prior to development of the assay was done at 24°C and 4°C and tested at days 1, 2, 4, 5, 9, and 10 post-collection. Working mixes w ere stored at 24, 4, and −20°C, and tested at weeks 1,2 and 3.

Reaction stability

Control assays were developed for 2hrs at 3 different temperatures (37°C, 24°C and 10°C) to determine whether the kinetics of the assay was affected by temperature. Given the identification of limitations related to storage at room temperature of blood-spots in filter paper previously reported [25], a selection of samples were assayed 24h after collection of the sample, and frozen at −20°C immediately after absorbance was quantified. They remained frozen for 1, 2, 3 and 4 weeks before G6PD assessment was carried out again both qualitatively and quantitatively.

Light

The 2hr development of assays for G6PD determination was done under various light conditions: in the dark, scattered light (indoors), and direct exposure to sunlight (outdoors).

Filter paper use

Assays for filter-paper saturation with blood-spots were done for both the WST8/1-methoxy PMS assay and the standard test, to assess whether or not significant differences in saturation could affect G6PD level determination. Such assessment was done by perforating 5–6 blood spots with different levels of saturation from the filter paper, and comparing the final quantitative readout.

For all measurements, the same preliminary experiments to those carried out with the WST8/1-methoxy PMS assay were reproduced with the standard reference test.

Sample size

The sample size was computed based on G6PD deficiency prevalence previously calculated by two independent studies in Kampala (16%) [31,32]. To validate the WST8/1-methoxy PMS method by comparison to the reference test with 80% power, the minimum number of samples calculated was 108. The final number of samples compared was 122, and a further 113 samples were evaluated by the WST8/1-methoxy PMS method alone.

Data entry and statistical analysis

Data regarding clinical evaluations, and G6PD assay outcomes were double-entered and validated. Visual analysis was done independently by two observers. Agreement scores between observers, G6PD level visual determination, and quantitative data were produced, and analysed with STATA version 11 (STATA Corporation, College Station, TX). For analysis of the use of the two tests, a contingency table was produced and sensitivity, specificity, PPV and NPV were calculated. A Receiver Operating Characteristic (ROC) curve was calculated. Potential characteristics that could affect G6PD deficiency assessment including gender, age, haemoglobin levels, and prevalence of anaemia were tested by univariate and multivariate regression analysis. Agreement between observers regarding qualitative G6PD activity levels by the WST8 assay, and the R&D reference test was determined by calculating a weighted kappa (Kw) value. A p-value < 0.05 was considered as statistically significant.

Ethics

Ethical approval to perform the G6PD assay validation was obtained from the London School of Hygiene and Tropical Medicine Ethics Committee (application no. 010/361). The use of human participant samples from the ACT PRIME study was under ethical approval of the Makerere University School of Medicine Research and Ethical Committee (no. 2010–108), the Ugandan National Council for Science and Technology (no. HS 794), the LSHTM Ethics Committee (no. 5779), and the University of California San Francisco (no. 006160).

Results

WST8/1-methoxy PMS test use in a field setting

Timeframe and temperature storage conditions affect assay performance

Bloodspot storage

Following collection, a random selection of 150 FPBS were stored at two different temperatures (4°C and 24°C), and assayed at various days (1, 2, 4, 5, 6, 9, 10) to determine optimal storage times before degradation of G6PD occurs and risk of misclassification increases. G6PD enzymatic activity could still be accurately assessed 10 days after sample collection with storage of blood spots at 4°C. Beyond this timeframe, the risk of misclassification increased (Figure 1a). For samples stored at room temperature, enzyme degradation occurred at a faster rate than previously reported [25], and classification at day 5 post-sample collection was not possible due to a high degree of misclassification (Figure 1b).

Figure 1 Enzyme degradation due to storage on filter papers. a) 150 filter papers with control blood spots with normal activity, moderate deficiency, severe deficiency, and no enzyme (100%, 30%, 10%, and 0%) were stored for up to 10 days at 4°C, and their activity measured at days 1,2,4,6,9, and 10. **b**) Samples were stored at room temperature, in the dark, for days 1–5, and the activity measured daily.

Assay mix storage

The stability of assay concentrated mixes was evaluated for a three-week time frame following storage at room temperature, 4°C and −20°C. Assays were t hen developed and ODs measured at time 0, and weeks 1, 2, and 3. Results for assay mixes stored at room temperature and 4°C were comparable to those obtained by Kuwahata *et al.* [25]. Results for assay mixes stored at −20°C yielded comparable results to those obtained using fresh mixes at all time points evaluated.

Assayed plate storage post-development

As the storage time of blood spots prior to assay was limited due to enzyme degradation, developed plates were re-assayed after initial assessment, after storage at −20°C for various time points including 24 hours, 1, 2, 3 and 4 weeks. Figure 2 shows that both visual and quantitative assessment of samples evaluated using the WST8 test was possible at all time points.

Figure 2 Temperature effects on storage of developed assays. G6PD activity was measured by the WST8/1-methoxy PMS test on fresh samples. The developed assay was then stored at −20°C for 24h, and 1–4 weeks.

Temperature and scattered light had little effect on G6PD classification and assay performance

Half-hourly kinetics of assays developed at 10, 24 and 37°C were meas ured and shown in Figure 3a. It was observed that G6PD level assessment and classification was not compromised across temperature ranges, although G6PD level assessment at 10°C was complicated (Figure 3a). In terms of assay sensitivity to aberrant colouration due to light, exposure of the assay to scattered light had little effect on abnormal colour development during a 2hr period, however, direct exposure to UV light led to aberrant colour development (Figure 3b).

Figure 3 Assay kinetics at various temperature and light levels. a) G6PD activity measured after 2hr development at 37°C, 24°C, and 10°C. Classification of G6PD values was possible at all temperatures, with 10°C showing the least variation Results repe ated 3x in duplicate ($p > 0.05$). **b**) Colouration development in reagents only, following 2hr incubation outdoors (exposure to sunlight), and indoors (exposure to scattered light). Aberrant colouration measured at the same wavelength as the G6PD assay (OD 450nm) was detected.

Test validation by comparison to reference test

High inter-observer reliability exists for qualitative classification of G6PD levels using the WST8 test

A weighted kappa statistic (Kw) for inter-observer reliability (based on qualitative G6PD classification by two observers visual assessment) was calculated to be 0.922, indicating excellent agreement. Paired assessment was conducted for 122 samples. Most mismatches between observers occurred for samples with G6PD levels with threshold values between mild deficiency (30-60% activity) and normal activity. Such range of G6PD levels is not of significant clinical relevance. A 90% agreement between both observers and the quantitative estimation of enzyme activity was calculated, and 10% discordance in samples with borderline G6PD values (at the normal/moderately deficient threshold) was found. Importantly, moderate and severe deficiency values were always accurately classified. It was observed that fresh human blood controls led to an estimation of a significantly higher percentage of G6PD deficient samples (in the 10-20% activity range), than the commercial control ($p < 0.05$). Controls with similar storage time-frames as the samples being tested were used, in order to prevent misclassification due to higher or lower reference OD values, as has been also reported elsewhere [25,30,33].

The WST8/1-methoxy-PMS test has high agreement with the reference test

Agreement values between the WST8/1-methoxy-PMS assay and the reference R&D test were assessed using the categorization of G6PD enzyme function into a) severe deficiency (<10% G6PD activity), moderate deficiency (10-30%), mild deficiency (30-60%), and normal activity (60-100%). Results are shown in Table 2. There was 100% agreement in classification of severe, moderate, and >150% activity samples. The lowest agreement recorded occurred near the cut-off point between normal and mild deficiency values (40-60% enzyme activity). Importantly, both the WST8 test, and the R&D test enabled identification of individuals with low G6PD enzyme activity with the highest risk for haemolytic anaemia (<30% activity). The Using the R&D test as a reference standard, the WST8 test's overall sensitivity for G6PD normal or G6PD deficient was found to be 72%, specificity 98%, PPV 91.3%, NPV 91.9%. The overall percentage of correct diagnosis was 91.8%, and an AUC value of 0.904 was calculated (Figure 4).

Table 2 Detection of G6PD deficiency levels: agreement and validation of WST8/1-methoxy PMS test

	WST8/1-methoxy PMS	Standard colourimetric test	Agreement (%)
Total samples tested	122	122	-
Normal activity	98 (80.4%)	94 (77.04%)	92.63%
Mild deficiency (30-60% activity)	15 (12.3%)	21 (16.4%)	96.72%
Moderate deficiency (10-30% activity)	9 (7.38%)	9 (7.38%)	100%
Severe deficiency (<10%)	0 (0%)	0 (0%)	100%

Table 3 Baseline measurements and G6PD classification by the WST8/1-methoxy PMS test

	Glucose-6-phosphate dehydrogenase classification				
	Normal (40-100 +% act.)	**Moderate (mild: 20-40% activity)**	**Moderate (low:10-20% activity)**	**Summary statistic (univariate normal/deficient)**	**Total**
Number					235
Males (n)	96	21	8	p = 0.136	125 (53.2%)
Females (n)	93	10	7		110 (46.8%)
Age (years, range)	2.83 (0.1-5.3)	2.89 (0.01-5.8)	2.87 (0.01 -5.1)	p = 0.802	2.84 (0.01 - 5.8)
Hb level (g/dl, mean, range)	11.18 (4.9-14.9)	11.74 (8–16)	11.90 (8.8 - 17)	p = 0.022	11.3 (4.9 - 17.0)
Anaemia (%)	28.57	16.12	20.0	p = 0.072	26.4%
G6PD activity (%, range)	72.6 (40.3-137.8)	29.2 (20.5-39.6)	15.11(10.3-19.8)	n/a	63.2 (10.3-137.8)

Figure 4 Validation of the WST8/1-methoxy PMS assay (AUC). Receiver operating characteristic curve for the performance of the WST8/1-methoxy PMS test for G6PD diagnosis in the field study in Uganda.

Study population and assessment of G6PD enzymatic activity

Samples from 235 children (110 females, 125 males) were analysed. No significant difference in G6PD deficiency levels between males and females was found (p = 0.136) by either the WST test or the reference R&D test. Among the male children, 16.5% showed intermediate levels of G6PD activity (Figure 5). Mean age and age distribution was similar among all G6PD classes (normal, mild, moderate and low deficiency) (p = 0.802). No severe deficiency was detected in this study population. While children with severe G6PD deficiency were not seen, G6PD values as low as 10.3% activity were identified. Anaemia prevalence (defined in this case as Hb levels under 10g/dl) was not significantly different between G6PD classes (p = 0.072). However, overall haemoglobin levels between the 3 main G6PD classes was significantly different (p = 0.022), with general haemoglobin levels being lower in G6PD normal children (Table 2).

Figure 5 G6PD distribution by gender. a) Among 110 females, 84.5% had G6PD levels ranging from 60% to 123% activity; 9.37% of females had G6PD activity lower than 30% - the activity threshold established by the WHO as posing a risk for primaquine administration at the present regime. Most females had activity values between 60 and 80%. **b**) Among 125 males, 76.8% had G6PD levels ranging from 40.3% to 137.8%. 9.4% of males had values lower than 30% activity. Most males had activity values in the 60-70% range.

Discussion

Susceptibility of G6PD deficient individuals to haemolysis caused by anti-malarial drugs such as primaquine and other 8-aminoquinolines is a concern for worldwide efforts for malaria eradication, given the geographical overlap between malaria-endemic areas and those populations with high prevalence of G6PD deficiency [12,13,27]. While primaquine administration without G6PD screening for confirmed malaria cases is thought to be relatively common, ethical issues regarding the use of the drug are regaining attention as wider community use is considered. At present, a main limitation for wide-scale implementation of G6PD screening is the lack of a robust, low-cost and rapid test that can accurately classify the majority of samples obtained from individuals in a steady state (ie. not suffering from haemolytic anaemia at the time of test), and that enables testing of a large number of samples simultaneously. In 2003, Tantular and Kawamoto published a simple screening method for detection of G6PD deficiency based on enzymatic activity, with improved performance and reagent stability compared to its predecessors [28]. Additionally, the method offers the advantage of enabling both qualitative and quantitative assessment of G6PD levels based on the NADPH concentration in the test, which yields strong colouration. Since its description in 2003, the assay has been used in various settings, including Thailand
[12] and Suriname [26]. In 2010, Kuwahata *et al.* successfully optimized the WST8/1-methoxy PMS assay for field use by adapting it to a 96-well plate format, and dried blood-spots in filter paper. The optimized test was successfully used to determine G6PD deficiency prevalence in Isabel Province, Solomon Islands [25]. Given the observations previously described regarding the performance of the WST8 test, the aims of this study were to validate the WST8 method in relation to a standard reference enzymatic test (commercially available

R&D); and to identify operational shortcomings and advantages of this test for use in field and resource-limited settings.

The assay was found to be easy to use, with low use of consumables, and low requirements for sophisticated equipment, as well as being less time consuming than the reference test. On average, processing time for a 96-well plate worth of samples took 10 minutes of active processing and a 2hr waiting period for development, while the R&D test took 1 hour of active processing. The WST8 test was not overly affected by temperature variation, and the temperature range within which accurate G6PD classification was possible, includes temperatures generally observed in tropical areas. Similarly, the test was less sensitive to scattered light in the laboratory than previously reported for other tests. Nevertheless, from our observations, we suggest avoiding unnecessary exposure of the test and the reagents to light for extended periods. In terms of storage of assay mixes and reagents, our conclusions are similar to those previously reached by Kuwahata *et al.* and confirm that long-term storage is advantageous for assay transport and assay use in field settings where assays may need to be run in the field, and subsequently tested in a central laboratory. This is likely, as a major limitation for storage is that enzyme degradation occurs in blood spots in filter papers limiting the time they can be held prior to testing. Previously, Kuwahata *et al.* determined that accurate G6PD classification could be done by the WST8 method on filter papers stored at ambient temperature for no more than five days, or alternatively at 4°C for up to 10 days. This is similar to the findings of this study for storage of samples at 4°C, yet storage of samples at room temperature for more than four days led to G6PD level misclassification. It is thus recommended that the samples be tested within 48-72h following sample collection, given that degradation time may vary slightly among different settings after this time frame. An alternative is the possibility to freeze assayed plates at −20°C for quantitation at a later time point. Although this requires freezing facilities, it would allow subsequent mass testing of samples for confirmation of visual readings. A key observation from this and previous studies [30], is that a control panel, which comprises positive controls and various levels of relative G6PD concentrations, should be stored in similar conditions to those of samples to be tested, as this will reduce the risk of misclassification. Two other observations, common to spectrophotometric assessments with FPBS were that both blood spot saturation and bubbles in the microplate wells can adversely affect reactions leading to aberrant readings and underestimation of G6PD levels. Overall, it was found that the WST8 assay offered major advantages in relation to other currently-used G6PD screening tests in its suitability for field use.

Importantly the assay also performed well. In comparison with the standard reference test, the WST8 test had 72% sensitivity, 98% specificity, and an AUC value of 0.904. The sensitivity of the test was only with misclassifications corresponding to samples with values between normal enzyme activity and mild G6PD deficiency i.e. individuals not at risk of severe haemolytic anaemia after treatment with primaquine (ie. >30% enzyme activity as defined by the WHO). Current tests, including the ICSH recommended fluorescent spot test (FST) method, report a sensitivity value as low as 32% [27,34-36]. In this context, the WST8 test enabled accurate identification of a wide range of G6PD enzyme levels. The study also showed a good inter-observer reliability (qualitative assessment) with very good agreement in relation to the quantitative classification though numbers of observers and samples were relatively few.

A known confounder for G6PD tests is haemoglobin concentration. This may be potentially attributed to the fact that in patients with haemolytic anaemia, older erythrocytes are

haemolysed, while the remaining reticulocytes have normal or near-normal enzyme activity. Previous G6PD screening studies have therefore suggested that G6PD testing must be done in parallel with haemoglobin measurements [25,33], or that inbuilt haemoglobin normalization must be considered for accurate determination of status [30]. In this study, baseline haemoglobin measurements in G6PD normal and G6PD-deficient children were significantly different by univariate analysis. The lower haemoglobin levels in children with normal G6PD in this study may be attributable to G6PD deficiency being associated with a protective effect against infections that may result in anaemia, however, the study was not powered to test this effect. Importantly, no severe deficiency was detected in this study population using both the reference and WST8 tests, which is in agreement with the expected G6PD A- prevalent genotype in Africa [5,8].

In conclusion, the WST8 test offers some important advantages in comparison to other tests for G6PD deficiency assessment in large-scale screening studies and public health interventions where primaquine administration is being considered. As demonstrated by this study, G6PD screening using the WST8 assay can be easily nested into other public health interventions, which is advantageous for its inclusion in malaria elimination programmes contemplating the use of primaquine. Additionally, the high comparability of quantitative and qualitative G6PD estimates between the WST8- and the standard colorimetric tests used for diagnosis in hospital settings, suggest that the WST8 test would be a relatively safe basis for clinical decisions. This would obviously depend on existing clinical and laboratory capacity in any facility and require some adaptation to single sample testing [37]. No individuals with severe deficiency were identified in this study. Although this is a limitation in terms of validation of the test, a previous study carried out in the Solomon Islands with the WST8 test [25], enabled the identification of severely G6PD deficient individuals, as well as a range of G6PD activities similar to the one reported here. In order to fully assess the capacity of the test in various field settings, further studies in various geographical locations where diverse G6PD genotypes are prevalent, would be advantageous. A further key observation is the need for parallel haemoglobin determination, emphasized in previous G6PD deficiency assessments [25,26,30,33]. This is likely to be of general benefit both in assessing the interpretability of the test and also may indicate other causes of anaemia and any required treatment. Overall, the WST8 test has a considerable potential as a diagnostic tool prior to primaquine administration in malaria-endemic areas as a point of care test and/or as a screening tool for assessing G6PD prevalence in large-scale screening studies in areas contemplating primaquine deployment.

Abbreviations

FPBS, Filter paper blood spots; FST, Fluorescent spot test; G6P, Glucose-6-phosphate; G6PD, Glucose-6-phosphate dehydrogenase; ICSH, International Council for Standardization of Haematology; NADP, Nicotinamide adenosine dinucleotide phosphate; NADPH, Nicotinamide adenosine dinucleotide phosphate reduced; WHO, World Health Organization; WST8/1-methoxy PMS, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt/ 1-methoxyphenazine methosulfate

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MDN, ACE, SS, CD wrote and edited the manuscript. MDN carried out the G6PD assay experiments and testing. MDN, ACE, CD carried out data analysis. CMS, DD, SG, PT and SS facilitated and supported the survey. MDN, EM, DN, LO, ES carried out sample collection and/or clinical assessment of the children in the study cohort. CD, ACE, and SS conceived the study. CD, ACE, SS, and MDN contributed to experiment design. ACE, SS, CD were involved in supervised field coordination. All authors read and approved the final manuscript.

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Figure 1

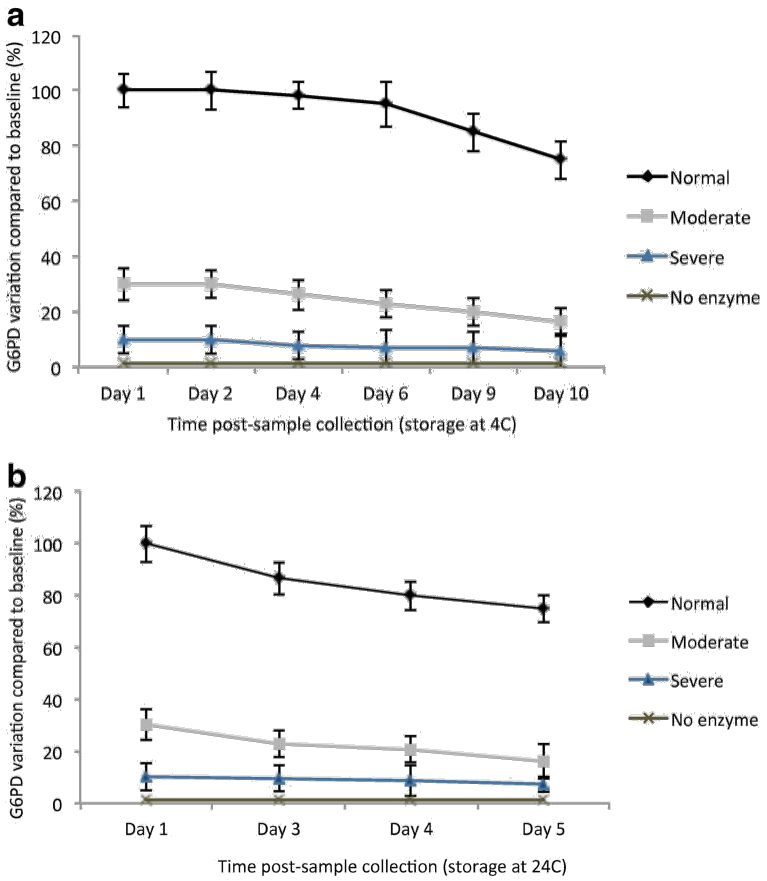


Figure 2

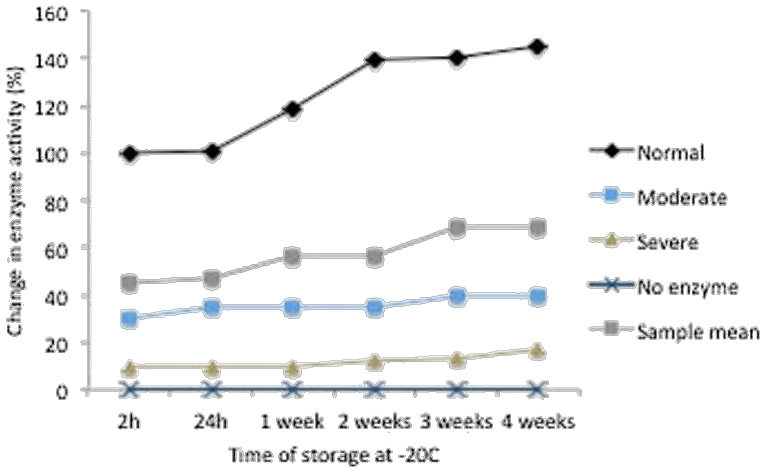


Figure 3

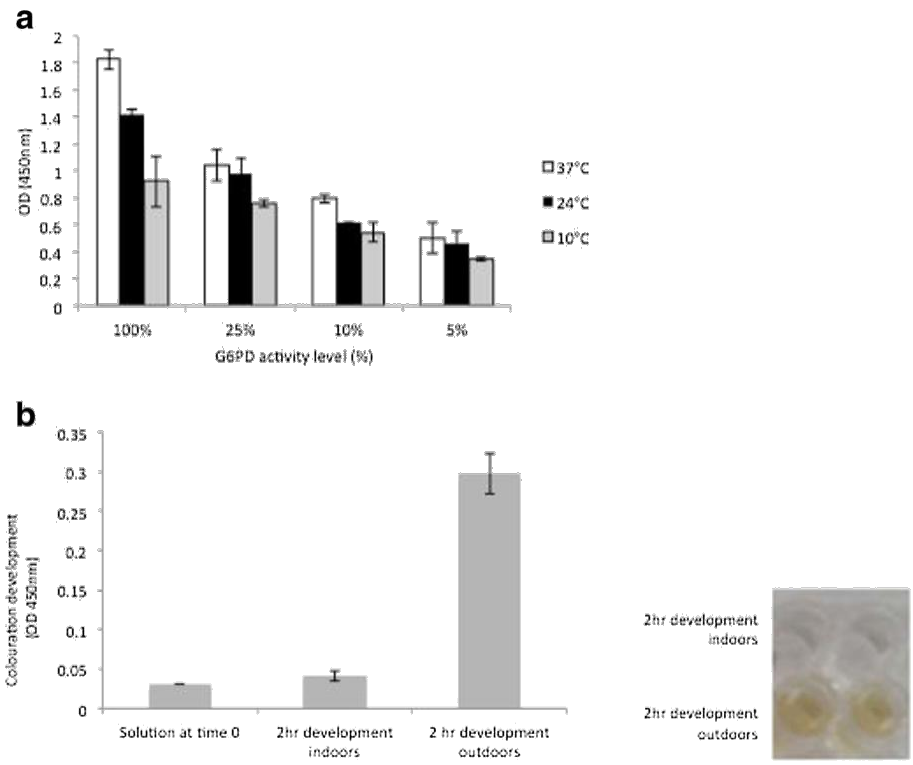


Figure 4

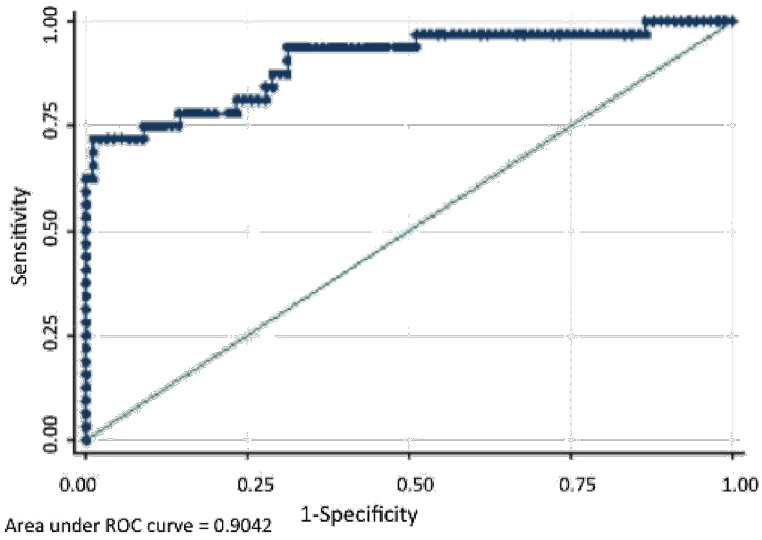
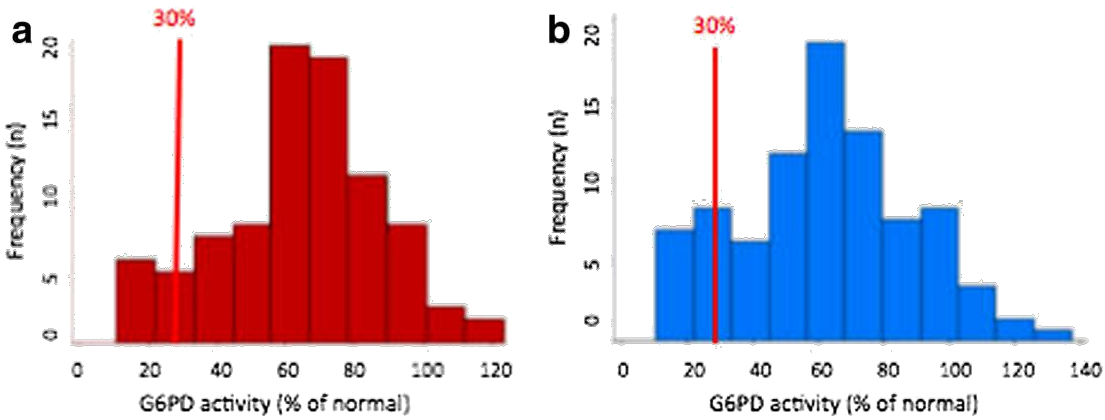


Figure 5


a
30%
Frequency (n)
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G6PD activity (% of normal)
b
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Frequency (n)
0
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140
G6PD activity (% of normal)

4.3 Additional sub-analyses of trial G6PD data

4.3.1 Assessment of G6PD status

4.3.1.1 *Assessment of G6PD phenotype by Fluorescent Spot Test*

Figure 4-1 The fluorescent spot test. Labelled blood spots from trial participants fluoresce under ultraviolet light after treatment with reagents.

The photograph shows a strip of filter paper with three labelled participant blood spots. In a darkened box. The strip is lit with ultraviolet light. Fluorescence under ultraviolet light is detectable in the second two blood spots, indicating normal G6PD enzyme level. The first blood spot remains dull, with no fluorescence, indicating a negative result (G6PD deficiency).

A positive fluorescent spot test (normal fluorescence) was a criterion for trial eligibility. 19.1% of the study participants with a normal spot test result had genotypic G6PD deficiency, with detectable G202A and A376G mutations (G6PD A- variant) (262).

4.3.1.2 *Assessment of G6PD genotype*

In the study, all individuals with the 376 A->G mutation had the 202 G->A mutation. Wild type individuals have 202G/376A in both X chromosomes. Individuals who carried both the mutation and carried the wild type sequence were labelled as heterozygotes (all females). Individuals who carried only the 202A/376G mutation were labelled as male hemizygotes or female homozygotes. The genotyping methodology and the breakdown of G6PD genotype by treatment arm are presented in a peer-reviewed manuscript (Section 4.1,) (262).

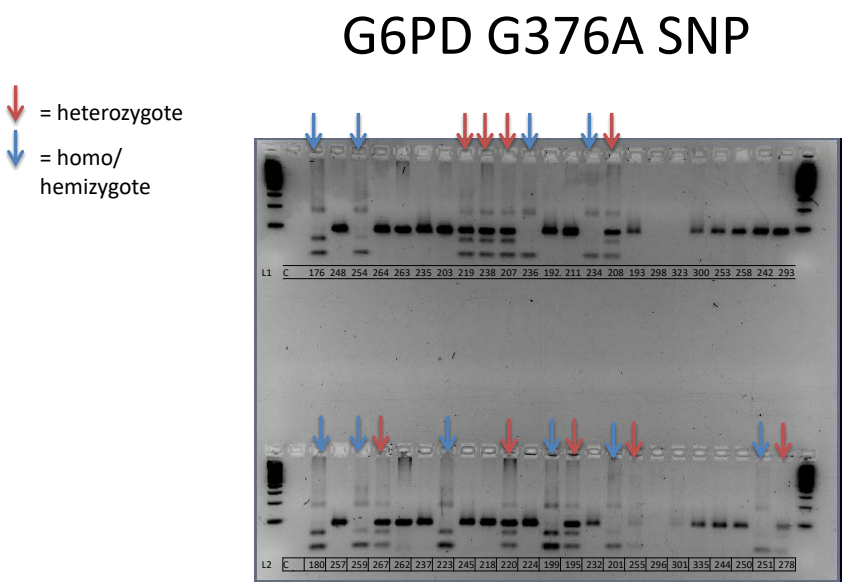

Figure 4-2 PCR-RFLP gel electrophoresis product for G6PD G376A SNP, courtesy of Dr Helmi Pett, University of Helsinki

Briefly, DNA was extracted from Whatman filter papers and was amplified in reaction solution containing primers for G6PD A- allele G376A primers, using BioTaq DNA polymerase. Amplified products were then digested with a restriction enzyme and analysed with gel electrophoresis (263, 264), with results as shown. The red arrows correspond to fragments from samples reacting with heterozygote primers and the blue arrows correspond to fragments from samples reacting with homo/ hemizygote primers. The first and last lanes in each row represent the base pair scale.

Higher baseline parasitaemias were found in female heterozygotes than in wild type or homo- / hemizygotes. Whilst there are hypotheses proposing that female heterozygotes are protected from severe disease (discussed in Section 4.4), further interpretation cannot be made with these results, as the study design and recruitment size were not planned for this evaluation.

Haemoglobin nadirs that occurred earlier in follow up might have been attributed to the haemolysis due to clinical malaria, combined with a lack of primaquine-induced haemolysis. A delayed nadir in haemoglobin might be due to the added haematological insult of primaquine dosing on day 2 of follow up in individuals who are susceptible to primaquine-induced haemolysis. Table 4-1 shows the day of haemoglobin nadir after enrolment. The nadir was earlier with increasing levels of G6PD deficiency, hemi/ homozygotes having earlier nadirs for a given primaquine dose arm, except for those receiving high dose primaquine (0.75mg/kg). In this group, the nadir was latest in those with G6PD deficiency. None of these trends reached significance; the numbers in these subgroups were small, and this sub-analysis was under-powered.

Table 4-1 Treatment day (after enrolment) of haemoglobin nadir by G6PD genotype across treatment arms

	Day of haemoglobin nadir after enrolment, by G6PD genotype								
	Wild type			Heterozygote			Hemi/homozygote		
Treatment arm	Mean	SD	P value*	Mean	SD	P value*	Mean	SD	P value*
AL	5.3	6.6	--	3.9	4.7	0.41	3.3	3.3	0.46
AL-PQ-0.1	4.8	5.6	--	2.8	2.5	0.14	2.3	0.5	0.25
AL-PQ-0.4	5.7	6.4	--	6.2	7.3	0.81	3.5	2.5	0.30
AL-PQ-0.75	4.2	4.4	--	5.9	4.3	0.19	6.3	3.8	0.36
All arms	5.0	5.8	--	4.5	4.9	0.56	3.6	2.8	0.22

**All P values are for the difference from wild type*

SD = standard deviation; AL = artemether-lumefantrine; PQ = primaquine

Only two people with a G6PD hemi-homozygote genotype had a maximal fall in haemoglobin of over 2g/dL during follow up, one from the placebo group and one from the 0.75mg/kg

primaquine group (table 4-2). This compared with 54 wild type and 17 heterozygote individuals. There was only one female homozygote, so determination of trends in the risk of haemolysis according to gender was not possible.

Table 4-2 G6PD status of individuals with a total fall in haemoglobin of >2g/dL during follow up

Primaquine dose	Wild type, n (%)	Heterozygote, n (%)	Homozygote	Total
Placebo	13 (24.07)	6 (35.29)	1 (50.00)	20
0.1 mg/kg	13 (24.07)	2 (11.76)	0	15
0.4 mg/kg	14 (25.93)	4 (23.53)	0	18
0.75 mg/kg	14 (25.93)	5 (29.41)	1 (50.00)	20
Total	54	17	2	73

4.3.1.3 *Assessment of G6PD enzyme activity*

The small total number of hemi-/ homozygotes limited the extent of meaningful sub-analysis of G6PD enzyme activity according to genotype. The data are presented here to illustrate trends, and generated hypotheses. Female heterozygotes had a broad range of enzyme activity levels, as would be expected due to lyonisation, but the median was significantly lower than wild type and homozygotes had uniformly less than 20% of normal male activity, corresponding with the significant change in haemoglobin from baseline in these individuals after higher doses of primaquine (262). Wild type individuals had a lower mean activity than expected.

Figure 4-3 shows the estimated G6PD enzyme activity, as a percentage of normal male activity grouped in bins of 10% and coded by G6PD genotype; values on the X-axis indicate the lower limit of these bins.

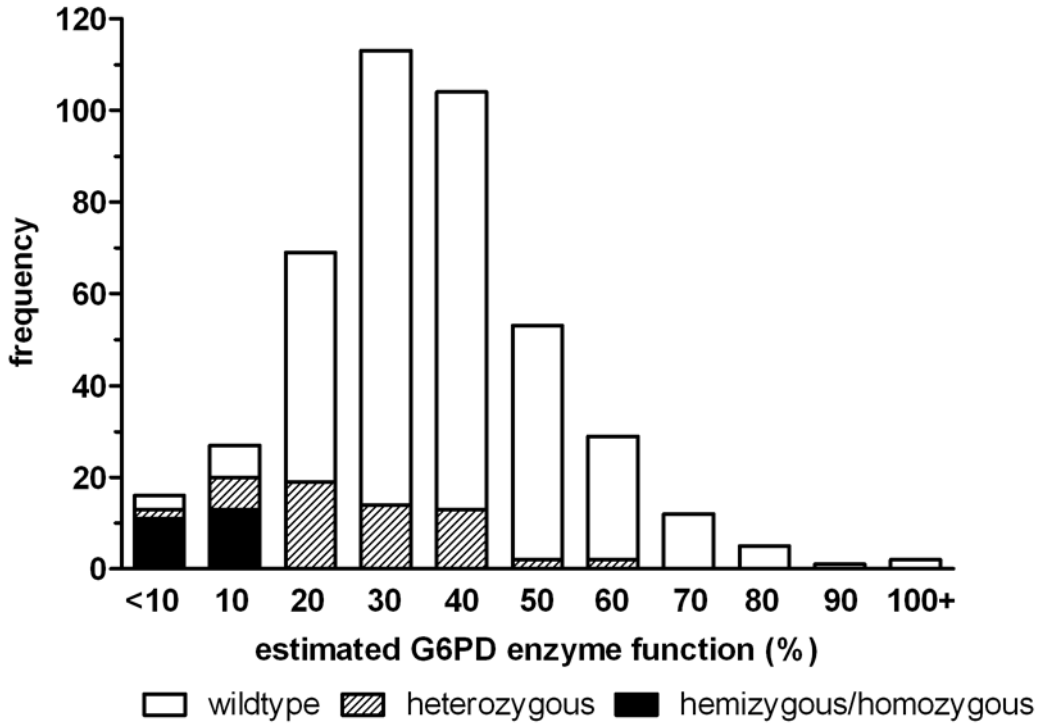

Figure 4-3 Estimated G6PD enzyme activity at enrolment in relation to G6PD A- genotype

Histogram of quantitative G6PD enzyme activity level shows the frequency distribution according to G6PD genotype.

The quantitative enzyme activity level at baseline (day 0), during clinical malaria infection, was compared to enzyme activity on day 14, when malaria parasitaemia and malaria-attributable fever was expected to have been cleared. This difference was found to be associated with G6PD genotype (p=0.044). Hemi-/ homozygotes, had significantly lower G6PD enzyme activity on day 14 compared to day 0 (Table 4-3, Figure 4-4). Wild type individuals had increased G6PD activity at day 14 as expected.

Table 4-3 Mean G6PD residual enzyme activity at enrolment and day 14, by G6PD genotype

	On Day 0 (enrolment)			On Day 14			Change	
G6PD Genotype	Total observations	Mean activity, % (SD)	P value for difference from wild type	Total observations	Mean activity, % (SD)	P value for difference from wild type	Change in activity, % (SD)	P value for difference from Day 0
Wild type	354	40.1 (16.0)	-	348	43.2 (16.7)	-	2.4 (18.6)	0.020
Heterozygote	58	32.5 (19.5)	0.001	59	31.8 (12.3)	<0.001	-1.7 (17.6)	0.45
Hemi/homozygote	26	16.1 (13.1)	<0.001	24	10.6 (3.5)	<0.001	-5.7 (13.4)	0.048

*residual enzyme function is % G6PD enzyme activity of normal male

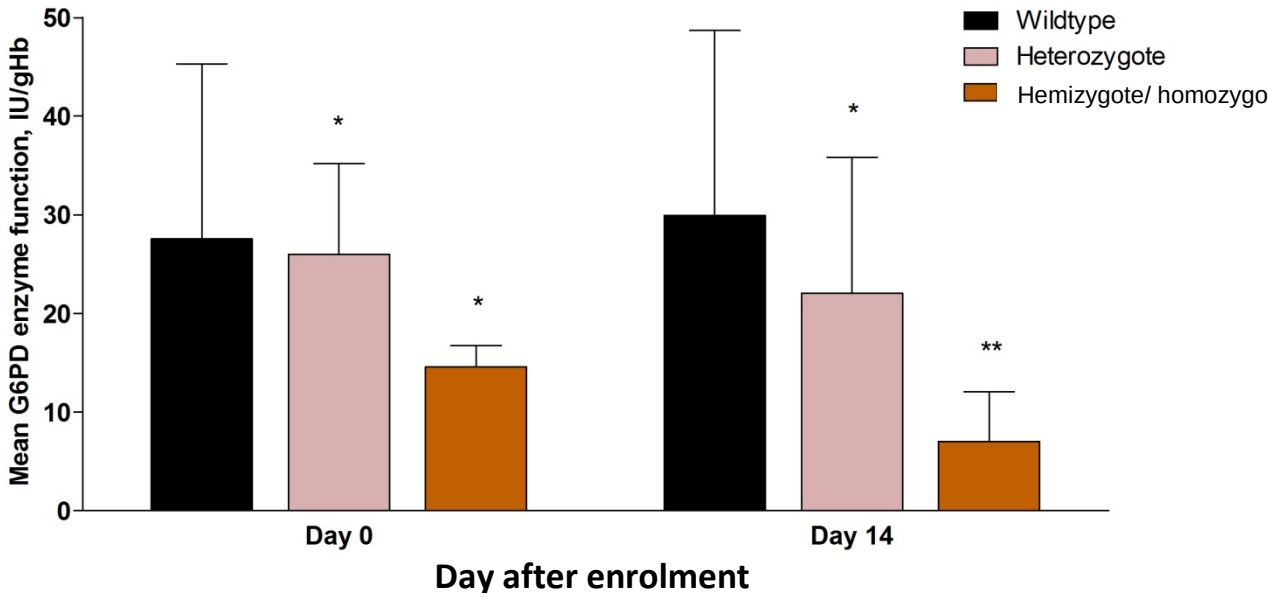

Figure 4-4 Change in G6PD enzyme activity during follow up

The mean G6PD enzyme function, as a percentage of a G6PD-normal male standard activity, is shown on day 0 (enrolment day) and day 14 after enrolment, stratified by G6PD genotype. G6PD enzyme activity in heterozygotes and hemi-/homozygotes was significantly lower than in individuals genotyped as wild type. This was the case at enrolment and on day 14. On day 14, G6PD enzyme activity had significantly reduced from enrolment values in hemi-/homozygous individuals.

**denotes significant difference from wild type; ** denotes both significant difference from wild type and significant reduction from Day 0*

4.3.1.4 *Definition of the optimal approaches for G6PD testing for the safe deployment of primaquine for falciparum malaria elimination*

In many regions of the world with overlapping *Plasmodium falciparum* and *P. vivax* malaria endemicity, particularly, in South and Southeast Asia and the Pacific, the obstacles to primaquine use have come under focus for both transmission-blocking (for *Plasmodium*

falciparum) and relapse prevention (for *P. vivax*) properties. A stakeholder meeting was held in Bangkok, Thailand in October 2012, to determine key research questions for the development of malaria and G6PD testing strategies and technologies, and priorities for product design for a range of use case scenarios and to determine relevant operational research priorities (185) (Appendix D).

There was consensus that there was an incomplete appreciation of the relationship between genotype and risk of haemolysis with primaquine dosing. The role of phenotypic testing both at the point of care and for population screening remained unclear, largely due to issues of test reliability, challenging logistical requirements of test kits, and lack of calibration to clinical outcomes. Regulatory issues and cost effectiveness were, at the time, undetermined. There was an incomplete picture of the range of genotypes prevalent in several malaria endemic areas, deeming population surveys an important research priority. Much discussion was held on the optimal enzyme function cut-off level for a test that would determine safe primaquine administration in those at risk of haemolysis and also that would avoid withholding primaquine administration (and the benefits of drug effect) in individuals who are not at risk. There was still a lack of clarity as to what was the level of haemolytic risk associated with single dose primaquine and whether G6PD testing would be required prior to treatment.

The meeting took place after this trial started, highlighting the lack of appropriate resources for G6PD testing in the context of primaquine use at the point of trial design, and the trial was part of the evidence base for the meeting. The aim of participation was to contribute to pushing the agenda to define what would constitute safe deployment of primaquine as a gametocytocide.

4.4 Discussion of G6PD sub-analyses

Much development has occurred in the evaluation of G6PD deficiency in relation to primaquine deployment since this trial completed and these are discussed in Chapter 5

(Discussion). In this section, there is a discussion of the sub-analyses conducted using the G6PD data in the trial.

Genotypic analysis of trial participants' blood samples showed that the fluorescent spot test failed to identify mild G6PD deficiency. There are several reasons why this may have occurred. First, it was predictable, given the low threshold for fluorescence ("normal" result) with the standard spot test assay that was used for exclusion from recruitment. Second, as female heterozygotes undergo lyonisation, their expression of the deficient gene is continuously variable and unpredictable so a proportion of females would be expected to exhibit a normal phenotype whilst carrying the deficient gene (182). Third, all of the participants had symptomatic malaria infection. Individuals with clinical malaria are expected to have a degree of haemolysis due to malaria infection, which drives their red blood cell population towards a left shift, or reticulocytosis, producing young, "fit" red cells with higher average G6PD function than the more aged red cell population in the non-haemolysing state. G6PD enzyme activity data from prospective community cohort studies sampling uninfected individuals is likely to be more reflective of baseline enzyme activity. Concomitant reticulocyte counts in this trial would have confirmed these assumptions. In some individuals, reticulocytosis may have occurred due to background co-morbidities associated with haemolytic anaemia that were not excluded by the selection criteria, such as undiagnosed beta thalassaemia, or other haemoglobinopathies, co-infections, drugs or auto-immune disease. Although a past medical history was taken to exclude these potential confounders as causes of significant morbidity, no diagnostic tests for these co-morbidities were performed at the point of screening or after recruitment.

The high degree of spread of enzyme activity data and the lower than expected activity in wild type individuals may reflect unreliability of the assay. As sample and assay kit storage conditions can affect the performance of the assay, efforts were made to control these

factors, including cool storage provision in the field laboratory, transfer in a portable refrigeration and freezer unit and installation of a back-up generator to enable continuous power supply during occasional national power cuts. Despite this, there were some inconsistencies in the cold chain. Therefore, these data were not presented as part of the peer reviewed publication. Two findings, however, show some expected trends. First, the female heterozygotes had a broad range of enzyme activity levels at the time of presentation with uncomplicated malaria (although it was, predictably, significantly lower than those with wild-type genotype), a finding which is expected due to lyonisation. Second, after recovery from clinical malaria on day 14, those with most severe enzyme deficiency, the hemi- and homozygotes, had significantly lower enzyme function levels than on day 0. This suggests that an initial malaria-associated reticulocytosis had masked their intrinsic enzyme deficiency at recruitment, and it explains why they had a normal spot test at recruitment such that they met inclusion criteria. Repeat fluorescent spot testing on day 14 or 28 may have confirmed this.

This raises an important consideration for future trial design and for the planning and implementation of community interventions. Phenotypic point-of-care G6PD testing may yield false negative (falsely normal) results in both female heterozygotes and in the context of acute clinical malaria. A given individual may express variable levels of G6PD activity depending on their physiological status at the time of sampling. Co-morbidity with other infections or injury may also affect production of reticulocytes (129). It is crucial to consider the immediate health status of participants when drawing inferences from G6PD analyses of trial data.

For community-level interventions, for individuals with acute haemolysis of any cause, false negatives are expected. If primaquine is to be administered with prior G6PD testing, as it is for radical cure (relapse prevention) of *Plasmodium vivax,* then future research needs to focus

not only on how to roll out G6PD testing for safe primaquine administration, but also on what tests are relevant in a given population, and how to predict the risk of haemolysis at the point of G6PD screening prior to primaquine administration.

As this trial data indicates, homo-/ hemizygotes and heterozygotes with falsely normal phenotypic G6PD screening results had a greater fall in haemoglobin after primaquine administration and this was dose-dependent. Although no individuals in the trial experienced severe haemolysis, when primaquine is administered at community level, a greater diversity of baseline haematological status and risk factors will be expected. The size of the risk in more susceptible individuals must be considered when extrapolating the predicted safety of a given dose of primaquine from clinical trial data.

In reality, both mass-testing for G6PD deficiency at the time of mass primaquine interventions, and individual-level testing at community health posts for case-based primaquine administration are expected to be logistically challenging and cost-efficiency is a major consideration (175, 187, 265). Hence, research policy for primaquine as a gametocytocide is targeted at determining a single low dose of primaquine that is predicted to be safe even in G6PD deficient individuals.

In conclusion, the G6PD genotype analysis from this trial indicates that 19% of children with uncomplicated malaria had a normal fluorescent spot test result (i.e., normal G6PD phenotype) on the day they came to the health centre with fever, but they had a genotype consistent with G6PD deficiency. The acute haemolysis of clinical malaria can mask their underlying G6PD deficiency. Other factors, including X chromosome lyonisation in female heterozygotes and co-morbidities that predispose to reticulocytosis may also have contributed to the false-negative fluorescent spot test results. Individuals who were misdiagnosed as having normal G6PD activity by phenotypic testing had a greater risk of primaquine-induced haemolysis. The elevated mean G6PD enzyme activity waned after the

transient reticulocytosis of acute malaria. For community primaquine interventions to be safe, the implemented dose of primaquine must be safe in asymptomatic G6PD deficient people, such as would be included in a mass drug administration or a mass screen and treat intervention (targeting asymptomatically infected people), who are not protected by a transient reticulocytosis at the time of dosing. Trials of reducing doses of primaquine in G6PD deficient males with asymptomatic *Plasmodium falciparum* malaria were conducted in Burkina Faso and the Gambia were designed following this trial, with contribution from this thesis, and the results indicate that low-dose primaquine is safe in this population (251).

5 Discussion

5.1 Statement of results

The World Health Organization recommends the addition of a single dose of primaquine to standard antimalarial treatment (ACT) as a gametocytocide, with the aim to block *Plasmodium falciparum* malaria transmission in the setting of malaria elimination programmes and as an intervention to stop the spread of artemisinin resistance (112). Since the 1960s, a single dose of 0.75mg/kg primaquine base has been recommended as a gametocytocide, but, historically, dose-finding studies for primaquine's efficacy for this indication have been marked by their absence. A reliable dose threshold for primaquine's safety was also lacking. Despite its incorporation into malaria guidelines for decades, the extent of primaquine's deployment was limited most likely because of concerns over the risk of haemolysis in people with G6PD deficiency.

This trial was designed in 2009, registered in 2010, and recruitment completed in 2012. It sought to address the evidence gap by providing relevant data on efficacy and safety outcomes in relation to primaquine dose for transmission-blocking. It was completed prior to a 2012 revision of the WHO guidelines on single-dose primaquine use, and it was the sole contemporary source of dose-finding data available at the time.

This first formal dose-finding trial tested the null hypothesis that non-inferiority of lower doses of primaquine for gametocyte clearance could not be established, compared to a reference dose of 0.75mg/kg; the dose that was recommended by the WHO at the start of the trial. Primaquine was administered in addition to the standard antimalarial treatment, artemether-lumefantrine, to 468 children in Jinja, Uganda with uncomplicated *Plasmodium falciparum* malaria and they were followed up for 28 days. The primary endpoint for efficacy was submicroscopic gametocyte clearance, and was measured by the mean duration of

gametocyte carriage, using QT NASBA for molecular gametocyte detection and was assessed for non-inferiority to the WHO reference dose. The primary safety endpoint, the mean maximal decrease in haemoglobin concentration over 28 days of follow up, was assessed for superiority to the placebo group who received artemether-lumefantrine alone (200).

The 0.75mg/kg dose reduced the mean duration of gametocyte carriage by 47%; it was 6.6 days (95% CI 5.3-7.8 days) compared to 12.4 days (95% CI 9.9-15.0 days; p<0.0001) in children receiving ACT alone (256). The rate of gametocyte clearance in the 0.4mg/kg group (6.3 days; 95% CI 5.1-7.5, p=0.74) was found to be non-inferior to the reference 0.75mg/kg dose, whilst the interpretation for the 0.1mg/kg group outcome (8.0 days; 95% CI 6.6-9.4, p<0.14) was "not non-inferior", or "inconclusive". None of the safety outcomes differed significantly from those in the ACT alone group.

Contemporaneously to the completion of this study, the WHO convened an expert review group to assess the safety and effectiveness of single dose primaquine as a *Plasmodium falciparum* gametocytocide. The expert panel reviewed historical studies and included this trial as the only contemporary dose-finding data available. The outcome was a revision of the recommended dose for transmission-blocking from the original 0.75mg/kg, recommended since the 1960s, to 0.25mg/kg primaquine base (257).

The historical data comprised small, non-randomised, transmission studies of between one to three individuals that pre-dated contemporary standards of research methodology (123). These studies assessed primaquine's transmission-blocking efficacy measured using mosquito feeding experiments and included only 10 participants that received the recommended dose of 0.25mg/kg or less (67). At the same time, primaquine's safety was reviewed in an extensive search of historical trials, largely non-randomised experiments on single individuals (110) or small numbers that predate modern standards of assessment. Safety data was also collated from post-implementation reports from mass drug administrations in the second half of the

20th century in China, Russia and North Korea and from an analysis of all reported deaths due to primaquine use.

At the time of study design, no clinical trials had been conducted to assess dose-finding for primaquine as a gametocytocide. Through the publication of its protocol, results and through data-sharing, this study has sparked a proliferation in research efforts to define and optimise the role of single-dose primaquine for *Plasmodium falciparum* transmission-blocking.

5.1.1 Limitations of trial design

The trial incorporated only four dose arms and none of these included the 0.25mg/kg primaquine base dose that the WHO selected for the revised guidelines. The 2012 WHO Expert Review Group meeting was initiated after this trial was designed and trial recruitment was almost complete. Given that there were only four dose intervals, statistical interpolation of these results to propose the lowest non-inferior dose was not undertaken. Instead, the new WHO-recommended 0.2gmg.kg dose was assessed by visual interpolation. The expectation that it would lie on a trajectory between that of the 0.1mg/kg and 0.4mg/kg primaquine base doses is illustrated in Figure 3-2 (Chapter 3). Following this trial, further clinical trials have adopted the published trial protocol (200) and incorporated the 0.25mg/kg dose arm.

5.1.1.1 *Can we translate drug efficacy into effectiveness?*

The primary efficacy outcome, the time to gametocyte clearance, was estimated by a mathematical model, using molecular quantification of gametocytes as an input. To use this data to inform malaria elimination policy, we must consider how variation in gametocyte clearance in an individual would translate to effectiveness at blocking malaria transmission at the community/ population level. Namely, what is the impact of a given reduction in the duration of submicroscopic gametocyte carriage in treated individuals upon the level of malaria transmission in the community? This question about effectiveness, rather than efficacy, has yet to be answered, despite the large number of clinical trials that are now

complete, or underway to further investigate primaquine as a transmission-blocker. The resources required to evaluate effectiveness at the level of malaria transmission in the community, using outcome measures such as community parasite prevalence and entomological inoculation rate (EIR), are expected to be considerable. In a large trial assessing the effect on community level transmission of a different control intervention designed to interrupt transmission (intermittent preventive treatment of school children with dihydroartemisinin-piperaquine), for example, the importance of high population coverage was acknowledged (266).

5.1.1.2 *Are we really measuring transmission-blocking?*

The ideal efficacy outcome measure for transmission-blocking intervention trials has yet to be defined, as is clear from the heterogeneity of trial methodologies (194). Mosquito feeding assays may be considered to be the gold standard in representing the biological outcome of infectivity to mosquitoes more accurately than gametocyte measurements (124, 199) but their utility for dose-finding has been hampered by the poor reproducibility and logistical complexity of these assays. Two approaches are used; skin feeding assays exhibit higher sensitivity, i.e., higher mosquito infection rates (267), but are not acceptable to ethical committees in many settings. Furthermore, they do not allow for quantification of the number of gametocytes or analysis of the constituents of the blood meal. Hence, there is no possibility for comparison of the infectiveness of different concentrations of gametocytes or evaluation of the effect of any relevant inhibitory factors in the blood the mosquitoes are ingesting, only in peripheral blood samples of the participant. Membrane feeding assays, by contrast, can be standardised, controlling the quantity, maturity and source of the feeding mosquitoes and the conditions and duration for their feed, but they are inherently variable both within and between sites. The likelihood that a reared mosquito will feed and that the ingested gametocytes will cause a mosquito infection is affected by the mosquito species and strain

(268, 269) and by variable mosquito factors such as the microbial flora of the mosquito midgut (270, 271) and probably by human immune responses to the parasite .

Delivering membrane feeding assays in the field is substantially more difficult than in optimal laboratory conditions. Membrane feeding is labour intensive and large numbers of mosquito feeds are required in order to maintain assay sensitivity. In Burkina Faso, a trial conducted in asymptomatically infected children used a similar protocol, but included membrane feeding assays to assess infectivity on days -1, 3, 7, 10 and 14 of follow up in a subset of individuals (259). In only one child was mosquito infection demonstrable after treatment; in the artemether-lumefantrine alone arm on day 7, the predetermined efficacy endpoint (259), leaving no scope to assess the impact of variable-doses of primaquine on transmission post treatment. In The Gambia, only two children infected mosquitoes 7 days after treatment; one in the placebo arm and one in the 0.2mg/kg primaquine treatment arm, in the higher dose primaquine arms (0.4 and 0.75mg/kg primaquine) there was no post-treatment transmission. In Mali, infectivity on day 7 was detectable in the control arm, receiving dihydroartemisinin piperaquine alone (3/13 individuals; 23%) and in two of the primaquine arms, 0.0625mg/kg (1/15 individuals; 6.7%) and 0.5mg/kg (1/14 individuals; 7.1%), but not in the intermediate doses (0.125mg/kg and 2.5mg.kg) (272). Analysis was limited to individuals who had a pre-treatment mosquito infectivity measurement and at least one post-treatment mosquito infectivity measurement. The membrane feeding assay was optimised after recruitment was initiated and the primary endpoint focussed on feeding on day 2 rather than day 7. The outcomes of membrane feeding on day 2 are discussed in section 6.3.1

Novel adaptations to the membrane feeding assay to allow higher throughput assays include the introduction of a transgenic *Plasmodium falciparum* “reporter” parasite that expresses luciferase, enabling detection of infected mosquitoes by luminescence readouts (273), but

currently their application is in screening for candidate drug compounds in vitro, not for clinical drug trials.

5.1.1.3 *What is the right gametocyte marker?*

Pfs25 mRNA has been used in a range of settings to detect and quantify mature gametocytes in field isolates (54, 63, 212, 274-276) . The molecular detection of gametocytes is up to ten times more sensitive than microscopy, with a detection limit of at least one gametocyte per microliter of blood (237, 277, 278). The precise mechanism of primaquine's action is unknown so the accuracy of any given mRNA marker in detecting the impact of primaquine, although it is gametocyte specific, is undetermined. There is some suggestion that primaquine may act earlier than *Pfs25* mRNA is expressed. Recently, *Pfs25* mRNA was found to be expressed almost exclusively in female gametocytes (279), whilst an alternative gene, *Pfs230p* (also Pf3D7/ PfMGET) mRNA appears to exhibit male-specificity (280, 281). Female gametocytes predominate in acute malaria infection, comprising approximately 70% of the circulating gametocyte population (124, 282), but the proportion of male gametocytes might relate more directly to the likelihood of infectivity (283). Male gametocytes appear to be more sensitive to certain antimalarial drugs than females (284), although this has not been determined clearly for primaquine. A recent paper suggests that primaquine does not preferentially clear male gametocytes (281). Amongst plasmodia species, the gametocyte sex ratio is found to vary during the course of an infection and with the degree of anaemia, reticulocytosis, asexual parasitaemia, and the density of gametocytes (282, 285, 286), reviewed in White 2014 (124).

5.1.1.4 *What is the point of counting gametocytes?*

The non-inferiority margin of 2.5 days revealed a dose-dependent effect that was reproduced in two subsequent trials using a comparable protocol (259, 287), but how well does 2.5 days of gametocyte carriage discriminate between effective doses to block transmission at community level? In the thesis, even with the highest dose of primaquine, six out of 106

children (5.7%) still carried gametocytes on day 14 after follow up. This figure was not significantly different for all primaquine doses in the trial; 3 out of 103 children (2.3%) with the non-inferior 0.4mg/kg dose (p=0.51), and 6 out of 103 children (5.8%) with 0.1mg/kg primaquine base (p=0.72) had a gametocytaemia on day 14. This prolonged persistence of gametocytaemia after primaquine in a subset of individuals has been noted subsequently (259, 287). To put this into context: children with uncomplicated malaria are managed as outpatients, so, the child treated with primaquine would return home from the clinic carrying gametocytes that are available for ingestion by biting mosquitoes in their home community. Whether these gametocytes are viable and of sufficient density to infect mosquitoes successfully is a pivotal question. Molecular detection of gametocytes using *pfs*25 shows a positive correlation with mosquito infectivity, but it is a weak and indirect trend (46, 124, 288). Mosquito feeding experiments suggest that primaquine renders gametocytes non-infectious within 24 to 48 hours of treatment (67, 77, 124). Gametocytes that persist beyond this timeframe may be non-viable, their duration in the circulation being determined by their rate of clearance by the spleen rather than any continuing drug effect. Recent work highlights that gametocyte density is independent of the transmission-blocking effect of primaquine in the first 48 hours after treatment (289). Hence, although gametocyte clearance did effectively discriminate between primaquine doses, and these dose-dependent trends have been reproduced in subsequent trials (259, 287), these trials might underestimate the size of the effect of primaquine on transmission blocking. Furthermore, the gametocyte prevalences post primaquine administration were low. This has important policy implications; the threshold dose for efficacy must be in line with the threshold dose for safety in G6PD deficient individuals. Clearly, high quality informative safety trials are needed to establish an effective dose range for primaquine deployment. Work towards this has been started with trials in West Africa and Myanmar (251, 290).

In interpreting the trial outcomes, we must take into account that a mathematical model was used to estimate the actual day of gametocyte clearance (200, 258). Gametocyte prevalence, assessed at a limited number of time points, was used to populate the model. The timescale for gametocyte measurements was limited to 14 days to reduce the confounding potential of reinfections, which would be expected to be more common thereafter (63, 258), but this also limited accuracy in extrapolating individual clearance times. The model incorporates an assumption to estimate the proportion of gametocytes that are released from sequestration. This might be affected by the trial drugs. Currently, we have no established method for assessing the sequestered gametocyte load nor how these gametocytes are affected by drug treatment and how their infectivity is affected upon release into the circulation (108, 258, 291-293).

5.1.1.5 *Does the trial setting matter?*

The trial was conducted in a moderate malaria transmission setting; the annual epidemiological inoculation rate in Walukuba was 3.8 in 2012 (294). How applicable the trial data is to other transmission settings is worth exploring, considering that primaquine is a candidate intervention in pre-elimination or elimination settings. Specifically, would the trial safety and efficacy be preserved in other settings? Not all countries are comfortable incorporating primaquine recommendations into their malaria elimination policies unless there is local trial data in their setting (295). Gametocyte dynamics vary across epidemiological settings (230) and marked differences have been observed in the duration of gametocyte carriage after primaquine treatment in different geographical locations (258). Pre-treatment patent gametocyte levels are identified as a significant predictor of gametocytaemia after drug treatment (228) and broadly increase with transmission intensity (37). Further factors may affect the response to treatment according to transmission setting; the clonal complexity of parasites in any single infection increases with transmission intensity (296). Clonality impacts the rate of gametocyte maturation and release during an infection

(297) and by implication, the prevalence of peripheral blood gametocytes at any measured time point. Efficacy and safety will also be affected by variation in human immune responses to gametocytes and vector factors between populations and the genetic variation in G6PD alleles between populations.

The feasibility of conducting a primaquine trial in an elimination setting, i.e., an EIR of less than one infective bite per person per year, is limited significantly by the low case incidence rate. In Zanzibar, for example, there are less than 1000 microscopy-confirmed cases per year (20). This is too few to enable completion of a well-powered clinical dose-finding trial both cost-effectively and to a timeline that would be useful to assist policy-makers intending to implement the WHO recommendations for primaquine use. For the purpose of dose-finding, therefore, we decided that a moderate transmission setting in East Africa would enable timely collection of relevant data. The choice to test primaquine in clinical cases and in individuals with higher densities of gametocytes was made in order to produce data about efficacy in individuals who are most likely to be the infectious (compared to individuals from elimination settings). We do not yet have data on how variation in the transmission setting might affect the trial outcomes.

5.1.1.6 *Are we collecting relevant safety data?*

Prior to this trial, no trials had been statistically powered to assess safety outcomes in individuals treated with primaquine for transmission blocking. Safety was assessed by passive pharmacovigilance of adverse events (298) or discrete haematological measurements in participants (63, 216) without assessment of the likelihood that a difference could be detectable between study arms.

The selection of an informative safety outcome is intuitive; given that primaquine induces haemolysis, an accurate measurement of haemoglobin levels post treatment was paramount. HemoCue®, a self-calibrating point of care test lends itself to the clinical trial setting. A recent

meta-analysis indicated that haemoglobin values measured by HemoCue® in 3084 patients diverged from gold standard laboratory assessment by 0.08 g/dL (95% CI -1.3, 1.4 g/dL) (299) and in children in the field, the correlation with gold standard was 98.7% ($p<0.0001$) (300). Only one trial prior to this had established a curve for the predicted fall in haemoglobin post single dose primaquine (63). In this study in Tanzanian children with clinical malaria, the haemoglobin nadir in the primaquine-containing arm was on day 7 (5 days after primaquine administration), whilst those who received ACT alone had a haemoglobin nadir on day 3. Clearly, there is an expected haemolysis attributable to clinical malaria and ACT and the additional impact of primaquine would be expected to depend on the severity of presentation, co-morbidities, gender and the level of functional G6PD enzyme as well as the dose of primaquine administered. In this thesis, we aimed to capture a range of dynamics of the haemoglobin response post primaquine: maximal fall, nadir day, percentage fall. An endpoint measuring haemoglobin at a single time point post treatment might not have captured differences in haemoglobin between dose arms. The day of dosing impacts the optimal scheduled days of haemoglobin measurement; if primaquine is given on day 0 rather than day 2, haemoglobin should be recovering by day 7 (63). We captured specific endpoints that would indicate severe haemolysis (requirement for blood transfusion, black urine) and any child whose haemoglobin fell below 5g/dl. These had not been captured in primaquine trials prior to this trial and have been incorporated into protocols subsequently. However, the reliability of these clinical markers for detecting severe haemolysis has not been quantified in a field context. Their detection depends on patient or parent reports and study clinician assessments. The decision to transfuse a patient depends on an assessment of their clinical status, not just on the level of haemoglobin. This is of particular importance when the level is measured by a point-of-care device (299).

An important limitation of this trial is that, in excluding children who were phenotypically G6PD deficient, the safety data cannot be used to predict the risk of haemolysis in children

with G6PD deficiency. As this was the first dose-finding trial, the ethical logic was to first assess the lowest efficacious dose of primaquine in a population at low risk of haemolytic side effects. Subsequent to this, trials were planned to assess the safety of this low dose in G6PD deficient populations(251).

5.2 The trial in context: updates since this trial (and trials in progress)

5.2.1 Primaquine dose-finding trials in Africa following this thesis

5.2.1.1 *Assessment of gametocyte clearance*

New trials that have assessed gametocyte outcomes after variable-dose primaquine have assessed different outcomes, and in different age groups and clinical populations but, in summary, have found the lowest efficacious dose of primaquine to be in the range of 0.2 to 0.4 mg/kg. In The Gambia, the primary endpoint of the fall in submicroscopic gametocyte prevalence between day 0 and day 7 in asymptomatically infected children after dihydroartemisinin-piperaquine was significantly greater for all three primaquine doses, 0.2 mg/kg, 0.4 mg/kg and 0.75 mg/kg (287). Gonçalves *et. al.* compared submicroscopic gametocyte prevalence after artemether-lumefantrine plus 0.25 mg/kg and 0.4 mg/kg primaquine with artemether-lumefantrine alone in 360 Burkinabe children with asymptomatic infection using superiority analysis (259). From day 7 onwards, for both primaquine doses, submicroscopic gametocyte prevalence was significantly lower than the control arm with and gametocyte clearance times were faster (7.7 days (6.3 – 9.1) for 0.25 mg/kg, p value <0.001 for difference from control; 8.2 (6.7 – 9.6) for 0.4 mg/kg arm, p value <0.001). In Mali submicroscopic gametocyte prevalence was significantly lower in the group receiving 0.5mg/kg primaquine base from day 7 onwards but gametocyte prevalence was not significantly different to control throughout follow up for lower doses (0.065, 0.125 and 0.25 mg/kg) (272). This smaller trial (n=79) was, however, not powered to assess gametocyte outcomes. All of these trials used superiority analysis to compare variable-dose primaquine to

placebo. To determine the lowest dose with equal efficacy to the well-investigated 0.75 mg/kg dose, non-inferiority analyses of these data would be informative, but would require larger numbers of participants.

5.2.1.2 *Assessment of infectivity to mosquitoes*

The adaptive trial design in Mali assessed the dose response to primaquine using the standardised membrane feeding assay as an outcome measure (272). The research team measured the reduction in post-treatment compared to pre-treatment mosquito infections in samples from 81 male participants (aged 7 to 32 years) who had *Plasmodium falciparum* gametocytes in their blood. No systematic process was employed to select participants for screening and they were a heterogeneous group in terms of malaria presentation; overall, 7% of participants had symptomatic uncomplicated malaria, ranging from zero in the control group to 19% (n=3) in the lowest dose group (0.0625mg/kg primaquine base), the remainder had asymptomatic infection. Participants were randomised to treatment with dihydroartemisinin-piperaquine alone (control) or in combination with a primaquine dose of 0.0625 mg/kg, 0.125 mg/kg, 0.25 mg/kg, and 0.5 mg/kg. Mosquito feeding was conducted prior to treatment on day 0, and also on day 1, day 2 and day 7. The primary efficacy endpoint was the mean within-person change in infectivity to mosquitoes, measured by comparing membrane feeding assay outcomes at baseline and on day 2. Significant reductions in day 2 infectivity were noted in the 0.25mg/kg and the 0.5mg/kg primaquine dose groups (92·6% [95% CI 78·3–100]; p=0·0014 and 75·0% [45·7–100]; p=0·014, respectively) compared to the control group (11·3% [–27·4 to 50·0]), but not for the lower dose groups of 0.0625mg/kg and 0.125mg/kg primaquine base. This supports the inference of the WHO primaquine expert review group, proposing 0.25mg/kg primaquine base as the lowest efficacious dose to block transmission of *Plasmodium falciparum* malaria (301).

Table 5-1 Completed contemporary primaquine dose-finding trials

Location	Author, date	Number of participants	Age	Malaria infection status	Primaquine doses assessed	PRIMAQUINE administered	Partner ACT	Gametocyte outcome	Infectivity studies	Safety
Gambia	Okebe (287)	694	Children	Asymptomatic	0.2, 0.4, 0.75 mg/kg	Day 2	DP	Day 7 vs day 0 prevalence	Day 7	Unpowered. Mean change in Hb
Burkina Faso	Gonçalves (259)	360	Children	Asymptomatic	0.25, 0.4 mg/kg	Day 2	AL	Prevalence and time to clearance	Day 0, 3, 7, 10, 14*	Unpowered. Mean change in Hb
Mali	Dicko (272)	81	Adults and children	Symptomatic (7%) and asymptomatic	0.0625, 0.125, 0.25, 0.5 mg/kg	Day 0	DP	Prevalence (and mosquito infectivity)	Day 1, 2 (primary outcome), 7†	Unpowered. Mean change in Hb
Uganda	Eziefula (256)	468	Children	Symptomatic	0.1, 0.4, 0.75 mg/kg	Day 2	AL	Prevalence and time to clearance	None	Powered. Mean change in Hb

**primaquine administered on day 2; †primaquine administered on day 0*

DP = dihydroartemisinin-piperaquine; AL = artemether-lumefantrine

5.2.1.3 *Assessment of safety*

Safety outcome measures have been more standardised across dose-finding trials (table 5-2), and have mirrored the protocol used in this trial, assessing the mean within-person change in haemoglobin over 28 days of follow up, using finger prick blood near-patient assessment (HemoCue AB, Ängelholm, Sweden). In trials in G6PD normal individuals, in asymptomatic Burkinabe children (259), in asymptomatic Gambian children (287) and in variably symptomatic Malian men and boys (272), the mean fall in haemoglobin was not significantly different in primaquine-containing arms compared to the control arm receiving ACT alone. None of these trials were specifically powered to assess safety outcomes and all of them included asymptomatic individuals. The risk of adverse safety outcomes is of particular pertinence in asymptomatic individuals receiving an antimalarial plus primaquine for the purpose of blocking community transmission, rather than the benefit of individual clinical cure. The fall in haemoglobin after ACT alone is expected to be smaller in the absence of malaria-associated haemolysis. Therefore, larger sample sizes might be required to discern any difference between treatment groups.

The recent dose-finding trials, for efficacy, in Africa have identified G6PD normal individuals, using phenotypic testing to screen for G6PD deficiency (259, 272, 287). It is not logistically practical to genotype individuals at the stage of screening for trial entry. Our finding, that 5.9% of phenotypically normal children were homozygous/hemizygous at the G6PD 202A locus (i.e., G6PD deficient of A- variant) and 13.2% were female heterozygotes demonstrates that these trial populations may include individuals at higher risk, unevenly distributed across dose arms (262). Enzymatic phenotypic testing for G6PD deficiency at the start of treatment, has reduced specificity compared with genotypic testing with regard to identifying those at risk of haemolysis. We found significant reductions in haemoglobin from baseline in the homozygous/hemizygous individuals (n=10) who received 0.4 mg/kg and in female

heterozygotes (n=14) who received 0.75 mg/kg primaquine base. The low numbers in each treatment group might explain the lack of trend of risk of haemolysis with dose.

Subsequent studies have assessed haemolytic risk in G6PD deficient populations. A study in Mali assessed single dose primaquine safety in 25 adult males and 26 male children aged 5-17 all with phenotypic G6PD deficiency (using R&D fluorescent spot test) and without malaria (microscopy negative) (302). Adults received doses of 0.4-0.5mg/kg primaquine base and children were treated with 0.4mg/kg. The largest within person fall in haemoglobin after primaquine was 23%, in an adult male who was one of the 40% of participants who had submicroscopic parasitaemia at enrolment and 8% who developed symptomatic malaria during follow up. The authors concluded that the upper bound of the therapeutic dose range for primaquine should be 0.4mg/kg in Africa.

In Burkina Faso and The Gambia, G6PD deficient male participants with asymptomatic malaria were treated with ACT alone or variable dose primaquine (0.25-0.4mg/kg) and post-treatment change in haemoglobin was compared with G6PD normal participants (251). No participants developed moderate or severe anaemia. The haemoglobin fall in G6PD deficient participants was greater than that in G6PD normal participants in Burkina Faso. Although 35-40% of all G6PD deficient participants across the two sites had a haemoglobin drop of >2.5g/dL, this was not statistically different to the fall in G6PD normal participants.

In Tanzania, a mixed gender population excluding pregnant and lactating women was treated for uncomplicated *Plasmodium falciparum* malaria with artemether-lumefantrine with and without a single dose of 0.25mg/kg primaquine base (303). The participants were enrolled regardless of G6PD status, but genotype analysis found a statistically significant greater day 0-7 fall in haemoglobin concentration in female G6PD heterozygotes compared to people with wild-type genotype.

All of these studies found a lack of severe haemolysis with primaquine doses of 0.5mg/kg base and below in G6PD deficient individuals. These are clinical trials with defined study populations. Primaquine is designed as an intervention for large unscreened populations. The common definition of severe haemolysis in these studies is a relative fall in haemoglobin post primaquine to >25% of baseline value. There is heterogeneity in these study populations in terms of malaria status (symptomatic, asymptomatic or malaria-free) and age, but crucial factors that affect baseline haemoglobin, such as co-morbidities, including HIV status, and presence of haemoglobinopathy (such as sickle cell anaemia, thalassaemia or haemoglobin C) are not considered in trial protocols or analysis strategies.

A more translatable evaluation was conducted in Thailand, forming the sub-analysis of a large community mass drug administration intervention with dihydroartemisinin-piperaquine plus single dose primaquine (0.25mg/kg base) (290). Bancone *et. al.* screened for eligibility to the sub-analysis using G6PD phenotypic testing, then further assessed G6PD genotype and quantitative enzyme function as we did. Of all those screened, four G6PD heterozygote women were misclassified as normal phenotypically and had significant falls in haemoglobin (either 25% fall from baseline or a reading less than 7g/dL) after primaquine treatment. Overall, as found in the African studies, the relative fall in haemoglobin after primaquine was greater in G6PD deficient individuals, by both phenotype and genotype, but there was no significant clinical haemolysis in any of the participants.

Table 5-2 Characteristics of safety trials of primaquine in Africa following this thesis

Location	Study ID	Number of participants	Age (yr)	Malaria infection status	Primaquine doses assessed	PRIMAQUINE dosing	Partner ACT	G6PD status	Safety	PK (Yes/ No)
Burkina Faso (251)	NCT02174900	70	18 to 45	Asymptomatic	0.25, 0.4 mg/kg	Day 2	AL	Normal and deficient males	Hb change 28 days	Y
Kenya (281)	NCT02259426	35/ arm		Asymptomatic	0.25 to 0.6 mg/kg	Day 2	DP	Normal and deficient	Hb change 14 days	N
Mali (302)	NCT02535767	28	18 to 50	Variable	0.4, 0.45, 0.50 mg/kg	Day 0	DP	Deficient males	Hb change 28 days	Y
Tanzania (303)	NCT02090036	220	>1	Symptomatic	0.25 mg/kg	Day 0	AL	Normal and deficient	Hb change 28 days	N
Swaziland, Senegal (304)	PROMPT (survey)	*	>1	Symptomatic	15 mg	Day 0	AL, DP, AS + AQ	Normal and deficient	Hb change day 0 to day 7	N
Gabon, DRC and 16 Asian sites	NCT02453308	1680	0.5 to 65	Symptomatic	Not specified	Day 0	DP, AL, MQ	Normal and deficient	Hb change 42 days	Y
Senegal (305)	PACTR201411 000937373	300	20 to 50	symptomatic	0.25 mg/kg	Day 0	ACT	Normal and deficient	Hb change day 0 to day 7	N

** pharmacovigilance study*

AL = artemether-lumefantrine; DP = dihydroartemisinin-piperaquine; AS = artesunate; AQ = amodiaquine; MQ = mefloquine

5.2.2 Alternatives to primaquine

5.2.2.1 *Existing drugs*

The efficacy and safety data we have available for primaquine in Africa is from well-defined low risk trial populations or from G6PD deficient individuals in a controlled environment. If we cannot guarantee primaquine's safety for general population roll out, where the risk in the context of co-morbidities, pregnancy or postnatal status, and potential drug interactions is undefined, should we opt for a safer alternative? If so, what drugs are available?

The thiazine dye, methylene blue was the first synthetic antimalarial compound (306). It is an inhibitor of the parasite glutathione reductase and, like the 4-aminoquinolones, prevents the polymerization of haem into haemozoin. *In vitro* schizontocidal activity has been demonstrated (307) and there is some evidence of synergy with the schizontocidal activity of artemisinin derivatives. It also prevents the development of methaemoglobinaemia; by converting iron from the ferric (Fe^{3+}) to its ferrous state (Fe^{2+}), it reduces oxidized haemoglobin. Its use as an antimalarial was phased out after the introduction and widespread use of chloroquine. Since then it has been used primarily as a treatment for pathological levels of methaemoglobinaemia. It is not effective as monotherapy, but there is renewed interest in its antimalarial properties as part of an ACT (308). Its gametocytocidal properties have prompted its investigation as an alternative to primaquine as a drug for malaria elimination.

Coulibaly et al compared gametocyte clearance after treatment of uncomplicated *Plasmodium falciparum* malaria in Burkinabe children with artesunate-amodiaquine with and without the addition of 15mg/kg (?base) methylene blue dispersible tablets (309). Gametocyte clearance was measured by reciprocal time to positivity (TTP^{-1}) of submicroscopic gametocytaemia,

measured by QT-NASBA. Despite the finding that a significantly higher proportion of children had microscopic gametocytes at baseline in the artesunate-amodiaquine plus methylene blue group (6.5% versus 1.0%, in the artesunate-amodiaquine group, p=0.04), by day 7, the TTP^{-1} was significantly lower in the artesunate-amodiaquine plus methylene blue group than the artesunate-amodiaquine alone group (0.037 [interquartile range 0.030-0.041] versus 0.045 [interquartile range 0.039-0.051], respectively, p<0.001).

Haemolytic toxicity is seen in individuals with G6PD deficiency (310). It can cause severe anaphylactoid serotonin toxicity in patients on monoamine oxidase inhibitors or selective serotonin reuptake inhibitors.

Following primaquine's introduction to malaria elimination strategies, methylene blue is being investigated as an alternative to primaquine. In a registered trial, NCT02851108, Burkinabe children aged 6 months to 5 years with any G6PD status and uncomplicated *Plasmodium falciparum* malaria received fixed dose artesunate-amodiaquine with either 0.25 mg/kg primaquine (n=50) on day 2 or 15 mg/kg methylene blue daily for three days. The primary endpoint was the day 0 to day 7 haemoglobin change and secondary endpoints included gametocyte prevalence and density assessed over 28 days. Compared to ACT alone, submicroscopic gametocyte prevalence was noted to be reduced with methylene blue from day 7 onwards in a previous trial but, significantly lower haemoglobin levels attributable to methylene blue were found on day 2 and day 7 after treatment started and there was significantly more vomiting in the methylene blue arm (309). A recent trial, in Mali compared primaquine and methylene blue with partner antimalarial treatment (311). Efficacy, safety and pharmacokinetics were evaluated in phenotypically G6PD normal males (aged 5-50 years) with asymptomatic gametocyte carriage in four parallel treatment arms. Sulphadoxine-pyrimethamine-amodiaquine was administered with and without 0.25 mg/kg primaquine on day 0 and dihydroartemisinin-piperaquine was administered with and without 15 mg/kg

methylene blue daily for three days. Transmission-blocking was assessed using gametocyte measurements and infectivity studies at baseline, day 2 and day 7. The two test drugs were given with different partner ACTs, which may have differing impacts on post-treatment gametocyte prevalence (section 5.2.3). Infectivity to mosquitoes was reduced significantly in the arms containing primaquine and methylene blue compared to their reference arms of partner drug alone. The transmission-blocking effect (using membrane feeding outcomes) and pharmacokinetics of primaquine versus methylene blue were explored further in an *in vitro* study in Thailand, NCT01668433. In summary, methylene blue appears to have a similar efficacy profile to primaquine. The haematological risk of methylene blue treatment also looks similar to that with primaquine dosing of 0.25-0.5mg/kg; a significantly reduced haemoglobin in the treatment arms but no severe haemolysis (309, 311). However, vomiting with methylene blue carried a risk of non-completion of treatment and exclusion from enrolment. This might make it a less attractive option for mass treatment.

Tafenoquine has been under evaluation for chemoprophylaxis for *Plasmodium falciparum* malaria (312) and for anti-relapse therapy for *Plasmodium vivax* infection (313, 314), but not for a transmission-blocking indication. The primaquine pro-drug bulaquine (synonyms: elubaquine, aablaquine) cleared microscopic gametocytes more rapidly and left fewer viable gametocytes compared with primaquine 0.75mg/kg base (215, 315). No trials are in the public domain comparing the safety and efficacy of bulaquine with low-dose primaquine. In particular, comparative mosquito infection efficacy data and safety data in G6PD deficient individuals may be of value.

Ivermectin is used for the treatment of nematode infections and scabies (316) and for mass drug administrations for the control and elimination of onchocerciasis (317, 318) and lymphatic filariasis (319). It has been found in *in vitro* and veterinary studies (320, 321), and in clinical studies (322, 323) to be lethal to anopheles mosquitoes (endectocidal) when ingested

in sufficient concentrations in a human or animal blood meal and this property has led to investigation of its potential as a tool for malaria elimination. For zoophilic Anopheline vectors, treatment of livestock may be optimal and for anthropophilic vectors, treatment of humans may have higher impact (324). Specific challenges include defining the endpoints to assess the efficacy of ivermectin, defining the optimal dose of ivermectin in humans for safety and efficacy to kill mosquitoes, delineating the strategy and dosing schedule in humans and livestock for its administration (325). In recent years, a research agenda has been developed to provide an informative evidence base (322) and a target product profile was generated by a technical committee and presented to the WHO Malaria Policy Advisory Committee (MPAC) to focus research and policy initiatives (326).

5.2.2.2 *New drugs*

Contemporary recommendations are that integral activity against sexual stages should be an essential characteristic, a component of the "target product profile", of all new antimalarial drugs (107). This has been accompanied by a flourish of high throughput methods to screen new candidate compounds for activity against gametocytes (108, 327-329). The development of stage-specific assays, for example, to assess in vitro efficacy, can highlight drugs with transmission-blocking effects as candidates for further development (108, 330, 331). Some examples are highlighted below.

The monovalent ionophores, including salinomycin, monensin and nigericin, are being repurposed from their established use in veterinary medicine (332, 333). They exhibit very low IC_{50} values for viability of mature gametocytes and ookinetes, as well as asexual stages, meriting further downstream drug evaluation (334).

In vitro assessments indicate that the spirindolone drug KAE609 (formerly, NITD609) significantly reduces early and late stage gametocyte counts and oocyst counts in standard membrane feeding assays (335). More recently, the drug has undergone phase II clinical trials

for both asexual and sexual stage efficacy against both *Plasmodium falciparum* and *P. vivax* malaria (336). Parasite clearance times are rapid and there are indications of good tolerability, nausea being the most common side effect. Participants were unselected for G6PD deficiency, given that drug-induced haemolysis has not been observed.

The imidazolopiperazine KAF156 has undergone Phase I (337) and Phase II trials (338). Slightly longer parasite clearance times are observed and adverse events of a range of character were seen in the majority of participants, the most common being sinus bradycardia, hypokalaemia, hyperbilirubinaemia, anaemia and thrombocytopenia.

5.2.3 The choice of partner ACT for combination with primaquine

Until novel compounds are available, which ACT should best be combined with primaquine for optimal effect on transmission? Following an ACT plus gametocytocidal drug intervention, a gradual resurgence of gametocyte carriage is observed in an endemic setting (after approximately 14 days following AL treatment) and is attributed to re-infection (63). Subsequently, the risk that individuals will be infectious will gradually increases (summarised in Bousema, 2011 (37)). An optimally-effective schizontocide should partner the gametocytocidal drug intervention in order to prevent emerging gametocytaemia from untreated asexual parasites. ACTs provide rapid and powerful asexual efficacy and have been recommended for first-line use globally since 2005 (339), but their effectiveness is threatened by the development of artemisinin resistance (22). Reduced asexual parasite clearance time was associated with patent gametocytaemia (above the microscopic detection level) after treatment in the TRAC study, that characterised and mapped artemisinin resistance across Southeast Asia and in three African sites (340). At sites with longer parasite clearance times, pre-treatment gametocytaemia was also more prevalent, suggesting a sustained effect at population level.

The choice of partnered drugs in an ACT is an integral determinant of its asexual efficacy (341) and failing antimalarial drug regimens have been characterised by increased post-treatment gametocytaemia (342) and the risk of onward transmission (66). A prospective study of 4116 children treated with four different ACT regimens for uncomplicated *Plasmodium falciparum* malaria across 12 sites in sub-Saharan Africa found gametocyte prevalence to be significantly higher in children after treatment with dihydroartemisinin-piperaquine or artesunate-amodiaquine or chlorproguanil-dapsone-artesunate than those treated with artemether-lumefantrine (343). The duration of gametocyte carriage was also shorter with AL.

A meta-analysis of 121 trials, including 48 840 patients confirmed these findings (48); sulphadoxine-pyrimethamine-amodiaquine and dihydroartemisinin-piperaquine were associated with an increased risk of development of patent gametocytaemia after treatment compared to artemether-lumefantrine of artesunate-mefloquine. This powerful analysis countered previous findings in showing no association of asexual parasite clearance times with post-treatment gametocytaemia, implying the importance of both the initial treatment efficacy and the post-treatment prophylaxis effect of the partner drug.

Will these differences in post-treatment gametocytaemia compromise primaquine's impact on transmission interruption enough to favour the selection of any given partner ACT? Primaquine's action against gametocytes is early and rapid and could be expected to negate the effects of varying ACT combinations. In Myanmar, there was no difference in patent gametocytaemia after treatment with six difference ACT regimens when 0.75mg/kg primaquine was added. With low dose primaquine, the assessment of microscopic gametocytaemia and transmission outcomes combined with different ACTs will be pertinent.

5.3 Areas for future research/ unanswered questions

5.3.1 Primaquine pharmacokinetics and pharmacodynamics

An important component of safe, widespread roll-out of primaquine is an understanding of the pharmacokinetics of the drug and its potential interactions with co-administered medications.

The cytochrome P450 isoenzymes, particularly 2D6, 3A4 and 2C19, play an significant role in primaquine metabolism, along with the monoamine oxidase enzymes (344, 345). Hence there is potential for interaction with other drugs. Similar to the synergy found between primaquine and chloroquine (346), increased plasma primaquine levels were found when it is co-administered with dihydroartemisinin-piperaquine (347). Primaquine concentration is also increased by co-administration with pyronaridine-artesunate (348).

In addition to CYP 2D6 enzyme activity, age and weight were found to affect primaquine pharmacokinetics in a sister study to this thesis (349). Unfortunately, due to substandard sample conditions in transit, all of the samples were thawed and, therefore, the novel pharmacokinetic analysis that was planned for this thesis (350) (Appendix A: Trial protocol) could not be conducted.

Primaquine is typically available in its racemic form. Recent work suggests that the different enantiomers vary in their anti-parasitic efficacy and also in toxicity in terms of propensity to cause methaemoglobinaemia and haemolysis (351). The different enantiomers are also metabolised at different rates, which may affect the likelihood of formation of clinically-significant metabolites (352). The properties of the primaquine enantiomers have been further characterised by population pharmacokinetic modelling (353).

Whilst polymorphisms resulting in differential isoenzyme activity may be more important in determining the outcome of longer course primaquine for *P. vivax* radical cure (354), these

findings need some consideration for single low-dose primaquine interventions. Clearly, more work is needed to establish any interactions between primaquine and commonly co-administered drugs, including anti-retroviral drugs for HIV.

5.3.2 Haematological response in asymptomatic and unparasitised populations

Reportedly, millions of people have received primaquine without screening for G6PD deficiency as part of large MDAs in the former USSR (355) , in China (87) and in US Army malaria programmes during the war in Vietnam (202) and Korea (87). All reported deaths due to primaquine have been associated with multiple doses (73). If single-dose primaquine is to be distributed in population mass treatment initiatives that incorporate asymptomatically infected or even uninfected individuals, a quantitative understanding of the risks is crucial. Very limited data are available currently.

In an extensive review of the safety of primaquine, Recht, *et al.,* (73) found that, in all records of patients receiving any dose of primaquine (single or multiple), whether in case-based treatment or mass drug administrations with published outcomes, the risk of death attributable to primaquine treatment was 1 in 621 428. For single low-dose primaquine , the risk of severity and death from haemolysis is expected to be low (290). Data are available from small, focussed clinical trials assessing the risk of haemolysis in G6PD deficient individuals (section 5.2.1.3 and 5.3.3), but these trials comprise a combination of symptomatic and asymptomatic individuals in a defined study population. Few trials have included unselected and untested individuals in a community.

5.3.3 Pharmacogenomic factors

The investigation of the haematological toxicity of primaquine in the 1950s led to the discovery of G6PD deficiency and highlighted the importance of pharmacogenomics in antimalarial therapeutics (95). The last few decades have seen increasing recognition of the role of genetic factors in treatment failures and drug-attributable adverse events. This has

driven research to increase our understanding of the molecular mechanism underlying particular phenotypes. There is a niche for pharmacogenomic analysis to help inform cost-effectiveness assessments and to help drive policy decisions (356). The Worldwide Antimalarial Resistance Network (WWARN) are collating and analysing huge pharmacogenetic datasets from clinical trials (see http://www.wwarn.org) to help define drug resistance and to optimise drug dosing. Regional bodies such as the African Medicines Regulatory Harmonization Initiative aim to facilitate processes to enable such data to impact policy and health (357) .

5.3.3.1 *G6PD variants*

Limited data are available on the safety of low dose primaquine in the wide range of G6PD deficient variants across the globe. Results are available from African studies assessing haemolysis risk with a single dose of 0.25mg/kg primaquine in G6PD deficient individuals (251, 302). These clinical trials involve well-defined study populations, but, few large community interventions have been conducted to assess primaquine safety in Africa, where the A- variant is prevalent. In a cluster randomised mass drug administration trial of 1110 individuals in Tanzania, given sulphadoxine-pyrimethamine plus artesunate plus primaquine (0.75mg/kg) versus placebo (358), although day 7 haemoglobin fell most significantly in the G6PD A- individuals, haemolysis was also seen in the wild type group and the most severe haemolytic event was in a child with G6PD B genotype (101). This suggests that other pharmacogenetic factors may be important, or that the single nucleotide polymorphisms used to identify the A, B and A- G6PD variants may incompletely define the range of G6PD alleles in the African population.

In Southeast Asia, compared to Africa, the diversity of genotypes is high, as is the range of residual enzyme function that the variants encode (191, 359). A large study of Targeted Malaria Elimination in a population on the Myanmar-Thailand border assessed the safety of

three monthly rounds of MDA containing dihydroartemisinin-piperaquine and a single dose of 0.25mg/kg primaquine (290). The frequency G6PD deficiency was 13.7% using phenotypic testing and residual enzyme function ranged from 3.9% of normal (Canton variant) to 73% of normal (Mahidol and Viangchan variants), with considerable variability seen within the Mahidol variant (290). The fractional fall in haemoglobin after treatment was significantly greater in G6PD deficient individuals after the first and second doses of primaquine and, unlike people with normal genotype, they did not see a total rise in haemoglobin over the three months of follow up. There were, however, no recorded episodes of symptomatic or clinically significant haemolysis, leading to the conclusion that low dose primaquine can be administered safely without prior G6PD testing.

The greatest unpredictability is in female G6PD heterozygotes, whose residual enzyme function may be highly variable (360). Effective prior testing at the point of care for G6PD status, particularly in this group, is challenging, because phenotypic tests may be normal. Further work correlating haemolytic risk with G6PD genotype, including exploration of new mutations using sequencing, and gender will be valuable.

5.3.3.2 *Cytochrome P450 (cyp) variants*

The isoenzyme cytochrome P450 2D6 is highly polymorphic with allelic variants exhibiting a broad range in levels of enzyme activity. In common with a significant proportion of drugs available on the market (361), it has an essential role in the hepatic metabolism of primaquine (345). In 2013, Bennet reported two failures of primaquine treatment for radical cure of P. vivax in two individuals in a malaria challenge experiment (362). They were found to have low CYP2D6 enzyme activity. Subsequent work demonstrated failure of primaquine as a causal prophylaxis in CYP2D6 knockout mice exposed to *Plasmodium berghei* infection (363), leading to concern that primaquine should be used with caution for primary prophylaxis in some human populations (364). CYP2D6 variants have been categorised into four different

phenotypes: poor, intermediate, extensive, and ultra-rapid metabolisers (365) . Potentially, identification of populations with a high frequency of poor metabolisers could highlight people at risk of drug failure. Ultra-high metabolisers might theoretically be at a higher risk of adverse events (344). However, thus far, it appears that the proportion of individuals with the extremes of enzyme functionality is relatively small (366) and although there is geographical variation in the distribution of genotypes, the diversity is higher within populations than between populations (366). At this stage, there is no obvious lead as to how to direct policy decisions in a given population. Whether there are settings where genotypic or phenotypic testing for CYP2D6 variation, for which a range of methods have been identified (367) will be cost-effective has yet to be determined. Testing requires large volumes of blood and high costs in terms of time, expertise and equipment. Furthermore, although CYP2D6-meditated metabolism is important for action against the hepatic stages of *Plasmodia spp.,* there is some evidence that it may be unnecessary for action against the asexual and the sexual erythrocytic stages (368). By contrast, CYP2C8, CYP2C9 and CYP3A5 activity appeared to correlate with *P. vivax* gametocyte clearance in 164 individuals in the Brazilian Amazon (369) after treatment with chloroquine and primaquine(369). Clearly, more pharmacogenomics work is needed in target populations for primaquine treatment.

Drug interactions mediated by CYP2D6 activity should also be considered, such as primaquine-chloroquine potentiation and the effect of other CYP p450 isoenzymes on the metabolism of ACTs (370). Pyronaridine, a relatively new antimalarial, is a potent inhibitor of CYP2D6, and increases the plasma concentration of primaquine (348).

247 out of 468 samples from the trial in this thesis were genotyped successfully for CYP2D6 (371, 372). The percentage of poor metabolisers and ultra-rapid metabolisers was 2%. 25% of the children were extensive metabolisers. For those who received the 0.4mg/kg primaquine dose, day 7 gametocyte prevalence was 7% (2/28) in children who were either extensive or

ultra rapid metabolisers, compared to 38% in the intermediate metabolisers and 100% (1/1) in the poor metaboliser (P=0.009). The unequal distribution of samples across treatment groups prevented further conclusive analysis. CYP2D6 activity data from this trial and subsequent primaquine trials in Burkina Faso, Mali, Kenya and The Gambia were pooled in a recent analysis (372). CYP2D6 data from these trials were incomplete, but suggested that poor and intermediate CYP2D6 metabolisers were more likely to have persisting gametocytes after ACT-primaquine treatment, whilst safety (haemoglobin concentration) was not affected.

The challenge is to decide what should be the policy implications for the size of the effect these polymorphisms have on primaquine safety and efficacy. Whilst these polymorphisms might be more relevant in determining outcomes of *Plasmodium vivax* anti-relapse treatment (373), currently, there is little suggestion that their effect should be taken into account in policy for *Plasmodium falciparum* gametocyte clearance treatment, given that the effect is comparatively limited.

5.3.3.3 *Methaemoglobinaemia*

Methaemoglobinaemia is an expected side effect of primaquine treatment (219). The oxidising action of primaquine increases production of methaemoglobin until drug levels fall and the NADH-dependent reducing system compensates and levels normalise (90). Methaemoglobin is seldom measured in primaquine safety evaluations, because after primaquine treatment, levels are typically sub-clinical (89). Levels increase in proportion with the dose of primaquine, but, even at a high dose of 1.14mg/kg daily for 14 day vivax relapse prevention, no treatment interventions were needed in a Colombian trial (374).

As the proportion of methaemoglobin in the blood increases, cyanosis is detectable, causing pale, grey or blue coloured skin, lips, and nail beds. Symptoms develop when methaemoglobin

levels are over 30%: light-headedness, headache, tachycardia, fatigue, dyspnoea, and lethargy. Very high levels, over 50-60%, may be life-threatening (90).

A cluster of congenital and acquired conditions can increase the propensity to methaemoglobinaemia. Congenital causes, such as methaemoglobin reductase deficiency (Cytochrome b5 reductase deficiency) may cause a raised baseline methaemoglobin or individuals may be asymptomatic unless exposed to an oxidising trigger (such as primaquine) (375). Acquired methaemoglobinaemia is precipitated only after ingestion of trigger compounds and may result from partial enzyme deficiency. Case reports have identified individuals with documented or likely enzyme deficiencies who have suffered clinical methaemoglobinaemia after antimalarial treatment (376, 377) . Future pharmacogenomic studies might elucidate the extent to which these polymorphisms contribute to primaquine-related adverse events.

5.3.4 The risk of primaquine in pregnancy and lactation

The G6PD status of the foetus cannot be determined routinely and therefore haemolytic or other risks due to primaquine treatment in pregnancy cannot be excluded. In accordance with the WHO guidelines and drug labelling, women who are pregnant have been excluded from population interventions and treatment with primaquine (33, 247). No clinical trials have assessed the efficacy or safety of single-dose primaquine for transmission-blocking in pregnant or lactating women. A missed abortion in a woman in Switzerland in 2002 was reported as potentially caused by the drugs she was treated with; primaquine and artemether-lumefantrine for malaria and ciprofloxacin, for a bacterial infection (73). Malaria infection itself increases the risk of stillbirth (378) and it increases the risk of miscarriage independently of antimalarial treatment (379).

A recent evaluation of primaquine pharmacokinetics in lactating women suggests that very limited amounts of primaquine are secreted in breastmilk and that the plasma concentrations

of primaquine in breastfed infants are too low to pose any risk of haemolysis (380). Plasma levels in women and infants were assessed during 14-day treatment for radical cure of *P. vivax* (primaquine base 0.5mg/kg/ day). The authors recommend that primaquine should not be withheld in breastfeeding women.

Pregnant or lactating women are likely to represent a significant proportion of the infectious reservoir for malaria transmission (381, 382) and the cost of their exclusion from community interventions must be considered, both in terms of operational feasibility and the effectiveness of the intervention.

5.3.5 HIV, malnutrition and other at risk populations

Primaquine administration in people who have both G6PD deficiency and an elevated baseline risk for anaemia would be expected to carry a higher likelihood of harm. Examples include HIV infection, helminth infection, malnutrition and chronic disease; conditions that are prevalent in malaria-endemic countries (383-386). HIV co-infection is known to increase the parasite density and severity of malaria and increase the risk of anaemia (387). In addition, the risk of drug-drug interactions from ongoing treatment, e.g. with anti-retroviral drugs for HIV, may increase the chance of adverse outcome. Co-morbidities are typically an exclusion criterion in clinical trials designed to assess the safety of low dose primaquine in African and Asian populations with G6PD deficiency (e.g., ClinicalTrials.gov trial identifiers: NCT02535767 in Mali, NCT02434952 in Cambodia, NCT02259426 in Kenya). Upon population deployment, however, it is in these vulnerable subgroups that the risk of primaquine treatment needs to be considered carefully. More research is needed to assess the likely impact of population interventions that would include these groups.

5.3.6 Endemicity: does the transmission setting matter?

Human-generated immune responses against molecular components of the gametocyte may affect the likelihood that the gametocyte develops to maturity, the rate of its clearance from the bloodstream and the success of its infectiousness to mosquitoes when ingested in a blood meal. They may also influence the likelihood of fertilisation and sporogony within the mosquito (388-390), (reviewed in (391)). These properties place them as candidate molecules for transmission-blocking vaccines (388). Their presence is correlated with reduced transmission in mosquito membrane feeding studies (392, 393).

There is some evidence that the expression of anti-gametocyte antibodies is increased during the transmission season (394) and correlates with exposure to gametocytes (395). Therefore, the prevalence of anti-gametocyte immune responses may vary depending on extent of prior exposure to the parasite and with transmission intensity. The transmission-blocking effect of primaquine in trials, such as this one, conducted in a non-elimination setting may differ to one involving participants from an area of lower transmission intensity if different levels of anti-gametocyte immunity affect drug efficacy. In line with evaluations of transmission-blocking vaccines (51) the impact of the level of anti-gametocyte antibodies on likelihood of transmission with and without primaquine deserves consideration. Human anti-gametocyte antibodies have been shown to reduce transmission in mosquito feeding assays, but the effect is complex (393). It is unlikely that it would have a significant effect on the efficacy of primaquine.

5.3.7 Application: is there a role for primaquine in mass drug administrations?

As a tool for malaria elimination, primaquine is recommended to reduce transmission at population level. The outstanding question is how should the drug best be deployed? Current guidelines recommend adding primaquine to treatment of clinical cases of malaria, i.e., case-based treatment. There is much debate over the potential impact of this strategy compared to

mass drug administration campaigns designed to interrupt community-level transmission over a defined time period. Mass drug administration involves administration of antimalarials at the same time to all members of a given population regardless of age and sex and malaria infection status. High coverage is a crucial determinant of the impact of an MDA (84, 396) and this requires community buy-in of acceptability, safety and efficacy of the intervention and efficient systems for implementation and monitoring; all of which are logistically challenging (397-401).

In the last half century, several mass drug administrations have included primaquine or another 8-aminoquinoline as a gametocytocide, but they form a highly heterogeneous group of interventions, leaving little consensus data with which to predict the effect on community-level transmission, the optimal strategy for implementation or the safety implications when primaquine use is scaled up. Almost exclusively, they comprise baseline and end-line surveys of parasite prevalence (396, 401), rather than integrally testing a hypothesis, incorporating a control arm or randomisation strategy. There is great variation in the drug regimen administered, in the choice of schizontocidal drug, the dosing regimen, the number of rounds of MDA, the malaria species targeted, the simultaneous deployment of vector control interventions and the selection and handling of individuals who were excluded from the intervention (e.g. children, pregnant women and people with G6PD deficiency) (396, 401, 402). A small number of interventions are in low and moderate transmission settings, where elimination efforts will be focussed (see public health section). Conclusions on efficacy or effectiveness of MDA depend on how and at what time interval after the intervention it is assessed, since the reduction of transmission after MDA is expected to be transient, with an ultimate return to pre-intervention levels (84).

Reviews of primaquine-containing MDA interventions have discovered no reports of deaths, prolonged hospitalisations or blood transfusions (73, 396, 401), yet many programmes and

studies lack safety assessments relevant to the risk of haemolysis with G6PD deficiency or systematic prospective pharmacovigilance methodology. Two successive WHO Evidence Review Groups have concluded that clear evidence is lacking for any benefit of the addition of single low dose primaquine to MDA regimens (402, 403). Further studies will address the incorporation into MDA of alternative transmission-blocking agents, such as ivermectin or methylene blue (404).

Kaneko reported the complete absence of microscopic parasite detection in a population on Vanuatu island in following 9 weekly administrations of chloroquine, pyrimethamine-sulfadoxine, and primaquine (0.75mg/kg) (405). Song and colleagues conducted a MDA in 3653 individuals in rural Cambodia, administering low dose primaquine (9mg adult dose, approximately 0.15mg/kg) with artemisinin-piperaquine (406). The ACT was given at baseline, with primaquine, then the primaquine dose was repeated every 10 days for six months, regardless of G6PD status. ACT treatment was repeated if the village parasite rate was >10%. There was a dramatic reduction in, but not elimination of, microscopic parasitaemia over the three-year study period. There was no comparator arm without primaquine. The safety analysis of the three-monthly rounds of MDA with dihydroartemisinin-piperaquine and a single dose of 0.25mg/kg primaquine (on day 1) given in an MDA in Thailand (290) are reviewed in section 6.3.3.1. There was no comparator arm without primaquine administration. Subsequent southeast Asian primaquine-ACT containing mass drug administrations have demonstrated early reduction in parasite rates with three consecutive monthly rounds in Myanmar (407), and three annual rounds in Laos (408). Although primaquine was well-tolerated, the population parasite rate rose after discontinuation. An Indonesian MDA showed no impact on malaria transmission of two to three rounds of primaquine and dihydroartemisinin-piperaquine treatment (409). In the low transmission-setting of Zanzibar, a two-round primaquine-containing MDA had no effect on PCR-detected parasite rate (410). In contrast, in the high transmission setting of Comoros, a high-intensity

MDA consisting of 3-monthly artemisinin-piperaquine with or without low-dose primaquine over a year, with 85-93% coverage, reduced malaria cases and malaria-attributable deaths (411). The effect on parasite rates was only reported in children, being reduced for up to 18 months post MDA.

Since mass treatment exposes community members who may be uninfected to potential drug toxicity, and the risk of emergent drug resistance, treating only those people who harbour malaria parasites may appear preferable. The cost-efficiency of pre-treatment screening for infection (mass screen and treat [MSAT] or focussed screen and treat [FSAT]) depends on optimised methods for the detection of infections and for implementation of testing. Screening with rapid diagnostic tests (RDTs) is found to miss low density infections (412-416) . Options include the use of more costly high-throughput PCR techniques (417) or opting for presumptive treatment with no screening.

An ambitious cluster randomised trial compared the impact on population parasite prevalence of focussed screening and treatment (FSAT or fMDA) with more standard MDA compared with no mass treatment and found no benefit from screening (with RDTs) prior to treatment (418). The ACT used was dihydroartemisinin-piperaquine and primaquine was not given. A clear benefit of MDA was offset by a parallel reduction in parasite prevalence and malaria incidence in the control arms, attributed to improved access to treatment and vector control during the study period. Further studies have found a limited impact of mass screening prior to treatment (413, 419, 420) and it was advised against in the recommendations of the WHO Evidence Review Group meeting (403).

It is clear that we need to understand how MDA can best be incorporated into long-term elimination strategies alongside other control interventions in order to sustain a lasting impact on transmission (25, 402). Current, evidence suggests that MDA antimalarial drugs

should have a long half-life and be different to the national first-line antimalarial treatment, to minimise risks of the development of drug resistance.

More work is needed to assess the relative contribution of primaquine (or other gametocytocidal/ transmission-blockers) versus ACT (or other schizontocide); to explore at what level of transmission and in what populations MDA is likely to have the highest impact; to determine how safety can be optimised and monitored; and to explore the relative effect of ACT drug efficacy, in the context of emerging artemisinin-resistant parasites. Table 5-3 highlights the settings where the WHO Evidence Review Group proposes that MDA should be considered for *Plasmodium falciparum* control (402) (Table 5-3).

Table 5-3 Settings in which mass drug administration should be considered for control of *Plasmodium falciparum* malaria, as advised by the WHO Evidence Review Group, 2019 (402)

Settings where mass drug administration may contribute to the control of Plasmodium falciparum malaria
Low transmission areas approaching elimination with good access to treatment, minimal risk of re-introduction of infection and implementation of vector control and surveillance.
Endemic island communities with limited risk of re-introduction of parasites, with implementation of effective treatment, vector control and surveillance
For short term reduction in transmission in areas of moderate to high transmission, but evidence is lacking that this accelerates progression towards elimination
To reduce the spread of multi-drug resistant malaria (in the Greater Mekong sub-region), but with recognition that effective antimalarial options for MDA are limited due to widespread multidrug resistance
As a time-limited intervention, to reduce morbidity and mortality where a health system is overwhelmed, such as, for epidemic control, and in complex emergencies

5.3.8 Dosing regimens for low-dose primaquine: single dose, multiple doses and seasonal dosing

The optimal timing of gametocytocidal interventions during the course of treating an infection has not yet been investigated. Given that ACT incompletely clears gametocytes (421), and considering the timescale of further emergence of gametocytaemia post ACT (WWARN gametocyte), how and with what treatment regimen can we get the greatest gains by using

primaquine to clear mature gametocytes? For optimal safety, dose-finding studies have administered primaquine on day 2, to avoid dosing during the haemolysis of acute malaria infection (63, 256, 259, 287), but day 0 administration retains high transmission-blocking efficacy (206, 422) and is operationally desirable, requiring no further interface with health services or reliance on compliance.

An open-label trial in 250 G6PD normal aged Tanzanian children aged 3 to 17 years with uncomplicated malaria will compare submicroscopic gametocytaemia and haemoglobin levels post dosing on day 0 versus day 2. 0.75mg/kg primaquine (NCT01906788).

Any residual asexual parasites after ACT therapy can potentially enable the development of mature gametocytes, leading to proposals that, for optimal impact, primaquine treatment should be repeated 2 weeks after ACT treatment (423). Where the efficacy of ACTs is reduced, with emerging artemisinin resistance, this may be a more important consideration, but operational practicality is likely to be challenging.

5.3.9 Modelling the effect of primaquine

Mathematical modelling provides a tool to predict the impact of health interventions. In the context of transmission-blocking, the lack of data on the community level impact of primaquine treatment leaves a niche for models to help guide policy. Models synthesise paradigms, using existing data as an input, to run simulations. The layering of different simulations can incorporate considerations of differing malaria epidemiology and differing intervention strategies (25, 424, 425), drug resistance and pharmacokinetics (426, 427) , parasite and vector biology (424, 428), health systems, treatment seeking behaviour and health economics (429) to increase the relevance to a given real-life setting (430, 431) . Where data are lacking, the models use assumptions and they often incorporate significant degrees of uncertainty; it is crucial that these limitations are considered carefully when using models to design interventions.

There is a consensus across modelling groups that the additional impact of primaquine, added to ACT interventions, will be small (432). The prediction is that ACT itself has such a large effect on transmission interruption, when administered at high coverage in a population, that ongoing transmission after a mass treatment intervention will be attributed mostly to individuals who did not participate and were untreated (432). The impact depends on whether ACTs are used for case based treatment, for presumptive treatment, or mass drug administration (25) Hence, although infectiousness after ACTs is well-demonstrated in individuals (37, 62-66), this is predicted to have only an incremental effect on ongoing transmission compared to reducing the asexual parasite burden by administering ACTs widely in the population (228, 426, 433). However, how primaquine's action is calculated in the models may affect the interpretation of their outputs. For example, data from gametocyte measurements underestimates the rapidity of primaquine's action compared to mosquito feeding assays (124). Furthermore, in the light of emerging artemisinin resistance, modelling suggests an important role for primaquine in preventing transmission of resistant parasites (425).

According to the models, two factors have a strong influence on the predicted impact of adding primaquine to ACT; the strategy for administration (to clinical cases versus to asymptomatic individuals) and the transmission intensity (434). Models evaluate primaquine interventions in two broad categories; primaquine administered to clinical malaria cases (25, 424, 435) and primaquine administered in mass treatment initiatives, i.e. to a defined population, regardless of symptomatology (402). Whereas most models incorporate addition of primaquine to treatment for clinical cases (436), there is some evidence that in mass drug administrations, primaquine can have a modest impact especially when combined with a drug with a long prophylactic effect, such as dihydroartemisinin-piperaquine (426, 434) .

Perhaps the most important factor determining the impact of primaquine is the duration of individual infectiousness. Conceptually, if individuals remain infectious for a short period, then primaquine may have more impact, but if the duration of infection is long, then primaquine is less likely to have any additional impact to a long-acting ACT. Certainly, in mass drug administrations, evidence indicates that ACTs with a long half-life are desirable for optimal effect (402). Findings that the choice of non-artemisinin partner drug affects the risk of post-treatment gametocytaemia (48) and by implication, the duration of infectiousness, make scrutiny of model parameters important. Primaquine may have a role in reducing post-treatment infectiousness with some ACT combinations more than others.

The consensus among modellers convening at a WHO expert review group in September 2015 was that available data predicts that incorporation of primaquine into MDA will have limited additional impact on transmission (432). However, subsequent to this, a role for primaquine as a transmission-blocker has been proposed in both case management and MDA in low transmission settings and in combination with an ACT with long-lasting prophylactic effect in high transmission settings (434).

5.3.10 Predicting and analysing the cost-benefit of low-dose primaquine

Cost-benefit analysis involves assessing the relative monetary costs of deploying an intervention, including the effects of any harms incurred and the costs to avert those harms, compared with the monetary benefits accrued from deploying the intervention, which may include the effects of prevented low productivity and deaths measured as disability-adjusted life-years (DALYs). A possible consideration prior to large scale intervention deployment, this has not been undertaken in depth for primaquine as a gametocytocide (197). The cost of excluding high-risk G6PD deficient individuals by prior testing needs to be considered. This has been explored for primaquine for radical cure of *Plasmodium vivax* infections (187, 437) and for the use of primaquine as a prophylactic agent (438). For *Plasmodium falciparum*

transmission-blocking, reliable prior testing for G6PD status in the individual healthcare setting in case-based treatment and in the field at population level for mass treatment initiatives would involve significant infrastructural costs to avert what appears to be a relatively small risk of significant haemolysis (Section 5.2.1.3). Identifying and excluding other high-risk groups, including pregnant women could be expected to have cost implications for both the delivery of the intervention and the benefit at population level. The accurate diagnosis, quantification and reliable recording of primaquine-induced haemolysis would be an essential component of a costed intervention and although tools have been proposed (304), effective systems for this pharmacovigilance have not yet been established. Although the cost of generic primaquine is small, accurate dispensing of the proposed low, single dose for transmission-blocking will involve the use of safely-prepared solutions as in this study or of new formulations of the drug, which might be expected come at an increased cost. It is hoped that these costs will not be prohibitive.

5.4 Public health application

5.4.1 What this trial addresses in the context of what is needed. Are we ready to use primaquine?

5.4.1.1 *Where and how should primaquine be used optimally?*

At the individual level, we have clear evidence for the efficacy of primaquine in reducing gametocyte carriage and blocking transmission to mosquitoes. Recent trials have further defined its safety profile (Section 5.2.1.3). The gap in evidence is in the translational aspects of primaquine's deployment. In what epidemiological settings would primaquine-containing interventions have the greatest impact? What is the optimal strategy for deployment; mass drug administrations, case-based treatment or a combination thereof? New research agendas will now focus on determining what sections of the population and what types of infection (symptomatic or asymptomatic) maintain transmission of *Plasmodium falciparum* malaria.

There is an important dichotomy focusing on the importance of treating the reservoir of asymptomatic infections. The proportion of submicroscopic and asymptomatic infections increases inversely with malaria transmission intensity, being highest in low endemic settings (415). In high endemic areas, asymptomatic infections predominate in individuals who have had most exposure to infections (30), typically, adults, who may be less easy than children to access in community outreach initiatives. One premise, in recognition of the burden of low density infections across the range of transmission settings, their adverse clinical consequences (439), the challenges of detecting them (412, 414, 419, 440), and their contribution to ongoing transmission (415), is that the greatest gains will come from population-wide mass-treatment interventions. The counter argument is that, despite their frequency, the actual contribution of low-density infections to ongoing transmission is small compared to that of patent, symptomatic infections and that targeting symptomatic infections, with optimised case-based treatment, even in the absence of a gametocytocidal drug, will reduce population transmission most efficiently (434). An advantage to this approach is logistical; as transmission intensity falls, clinical cases are more easily detected if adequate surveillance is in place, and strengthened health systems allow for prompt treatment. This may be particularly efficient when malaria transmission has reduced recently (441). The malaria elimination in Sri Lanka is a case in point. The successful elimination campaign was based largely on enhancing the standard of care of clinical cases and a focus on maintaining the supply chain of ACTs and rapid diagnostic tests (442). If primaquine is to be deployed, notwithstanding the inherent safety issues of its use, any success depends on treating the reservoir of infections that sustains transmission most efficiently in the population. Identifying the source and dynamics of ongoing transmission in any given population is therefore a priority for on-going research for malaria elimination.

5.4.1.2 *Pharmacovigilance: How can we ensure safety when primaquine is rolled out?*

Clinical trial evidence and extensive literature reviews suggest that low dose primaquine is safe, even in G6PD deficient individuals, but outside of the well-defined trial population, prediction of the risk to vulnerable members of the population is challenging (Section 5.3). Most studies reporting primaquine's safety in risk groups include a cut-off defining severe haemolysis as a fall in haemoglobin of >2.5g/dL or >25% fall from baseline value. In a large community roll-out of primaquine, how acceptable would this definition be in people who are anaemic at baseline due to co-morbidity? How acceptable would this risk be compared with other largely accepted risks, such as that of sulfadoxine-pyrimethamine-induced Stevens-Johnsons syndrome or toxic epidermal necrolysis? Large, historical mass drug administrations have reported are remarkable in the absence of reports of adverse events, haemolytic or otherwise associated with primaquine use (73, 401), but crucially, descriptions of surveillance systems for safety are limited. For a large roll-out of primaquine, tailor made pharmacovigilance would be essential. This would require large-scale health worker training, and provision for community-based G6PD screening, monitoring systems to detect severe haemolysis and platforms for data collection to monitor adverse events, potential drug interactions and safety in pregnancy and co-morbid conditions (443). The Primaquine Roll Out Monitoring Pharmacovigilance Tool (304) is being piloted in Swaziland in parallel with the implementation of policy to treat all clinical cases of malaria with ACT plus low dose primaquine. Safe deployment will require infrastructure to enable follow up and testing of haemoglobin and G6PD function in those at risk and access to safe and timely blood transfusion. This brings into question the cost-effectiveness of such an intervention; analyses that will need to be done to enable the process of policy development. A safer alternative to primaquine is clearly desirable although, as yet, none such exists. Pharmacovigilance infrastructure will be important for the range of transmission-blocking interventions under current evaluation, including tafenoquine and methylene blue.

5.4.1.3 *Therapeutic dose range*

At the country-level implementation, accurate primaquine dosing is a challenge. The need to titrate each dose to weight and the lack of incremental tablet sizes or a paediatric formulation mean that delivery of the drug in elimination interventions and in health clinics will require tailored equipment and training.

A therapeutic dose range for single dose primaquine for transmission blocking represents the lowest efficacious and the highest safe dose. More data from safety trials and a WWARN analysis is in process, of pooled data from primaquine trials will define this further (198). Accurate dosing by weight is feasible in a clinical trial setting, but is costly and highly challenging at a programmatic level. Age-based dosing is preferable, but must be designed to avoid over- or under-dosing children at the peripheries of each category. Hypothetical dose bands have been generated from modelling potential therapeutic ranges (260). A model incorporating pharmacological, pharmacokinetic and anthropometric data proposes four age bands for Cambodian children (444). A five age-band model has been developed for children in sub-Saharan Africa (445). Ideally, the dosing age-bands for primaquine will align with those for partner ACTs so that health workers can more easily assign as number of tablets for a given child's age. Currently, the smallest available tablet size is 7.5mg and the case is being put forward to manufacturers for production of smaller dose per tablet for children; the ideal tablet size being informed by dose modelling (250, 446).

5.4.1.4 *Drug availability/ licencing*

In the series of meetings of the Single Low-dose Primaquine Working Group, country representatives shared that they were encountering significant difficulty with the procurement of primaquine (443).

Drugs that are rolled out at scale by international procurement agencies must carry a high standard of globally-recognised regulatory approval (447, 448) typically requiring that they

have attained WHO pre-qualification. The process of WHO pre-qualification scrutinises thoroughly both the product, in terms of its safety, efficacy and quality, and the manufacturer, in terms of their ability to assure the quality of the product and ensure that this is maintained between batches (449). If WHO pre-qualification has not yet been attained, Stringent Regulatory Authority approval may be accepted instead. A SRA is a drug regulatory authority defined by the WHO as either: "a member of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (as specified on www.ich.org); or an ICH observer, being the European Free Trade Association (EFTA), as represented by Swissmedic and Health Canada (as may be updated from time to time); or a regulatory authority associated with an ICH member through a legally-binding, mutual recognition agreement including Australia, Iceland, Liechtenstein and Norway" (page 147 of (449)).

For the off-label indication of transmission-blocking, primaquine phosphate is neither WHO pre-qualified nor is it approved by an SRA. Since it is a generic drug, and its use is already endorsed by the WHO, it is unlikely that manufacturers of primaquine will seek a label-claim for the transmission-blocking indication (199). Therefore, the evidence base for primaquine for transmission-blocking will come from non-commercial investigators, such as was the case in this thesis. As has happened with precedents such as the programmatic use of ivermectin and albendazole for helminth infections, it looks likely that collaboration between the WHO and partners such as the Medicines for Malaria Venture and manufacturers will enable WHO approval for the off-label use of primaquine for transmission-blocking (443).

A recent survey found that, globally, only two manufacturers produce SRA-approved primaquine phosphate (Sanofi in Canada and Remedica in Cyprus), for the indication of radical cure of vivax (260). This mismatch between licensed indication and the need for high quality supply has meant that primaquine procurement is a logistical roadblock for countries

intending to put the current WHO guidelines on the use of primaquine for transmission-blocking into policy and practice (260, 446).

The Single Low-dose Primaquine Working Group (Section 3.3.4.1) created a platform to bring together stakeholders from procurers, the WHO and manufacturers such that agreements are now being forged to facilitate procurement for this indication (443).

5.4.1.5 *Countries using it already*

Prior to this trial, of the African countries targeting malaria elimination, none had incorporated primaquine use as a gametocytocide into national guidelines. In Ethiopia, primaquine was used with chloroquine for both *P. vivax* and *Plasmodium falciparum* malaria for 25 years until 1990, when its use was discontinued (199). Primaquine use as a gametocytocide alongside antimalarial therapy was adopted mainly in countries in South-East Asia and South America. These are regions where *P. vivax* and *Plasmodium falciparum* malaria co-exist, so there was established use of primaquine for vivax anti-relapse treatment as well. There was likely to have been a mismatch, however, between guideline-recommended use and actual use, particularly in Asia, because of safety concerns with G6PD deficiency (175). A limited number of mass drug administrations had incorporated primaquine with the aim to eliminate malaria (73, 83, 406, 411, 450, 451) .

Now that a lower dose has been authorised by the WHO, its use is more widespread. The World Malaria Report 2016 identified 31 countries that include low-dose primaquine in first line therapy of confirmed uncomplicated *Plasmodium falciparum* malaria in national guidelines (20), these are principally in South America, South-East Asian and Eastern Mediterranean regions. In 2017, this figure rose to 54 malaria-endemic countries (1). In 2015, African countries that expressed an intention to incorporate single low-dose primaquine into

policy included Ethiopia, Senegal, Zambia, Swaziland and Zanzibar (260). Since then, 14 African countries, largely those on the threshold of malaria elimination or pre-elimination, have adopted single low-dose primaquine into policy (1).

Primaquine as a *Plasmodium falciparum* transmission-blocker has been firmly incorporated into global policy and into implementation strategies for malaria elimination (452).

6 Conclusions

6.1 Global malaria control status: global targets and the role of primaquine

In the period 2010 to 2015, the global estimated number of malaria cases fell by 14% to total 212 million. There were 429 000 deaths, 92% of which were in Africa and 70% were in children aged under 5 years. Since 2015, the WHO Malaria Global Technical Strategy is to eliminate malaria from 35 endemic countries by 2030, and from 10 by 2020 (13). Single low-dose primaquine is now recommended as a component of the toolkit for countries and programmes targeting malaria elimination, and it has been incorporated into first-line treatment in 31 countries and its use is now recorded as an indicator of malaria policy adoption (20).

Although single low-dose primaquine is recommended widely, its case-based use is estimated to be more limited in regions with higher prevalence and severity of G6PD deficiency (175).

6.2 Contribution to knowledge

This trial contributed the first randomised and controlled dose-finding data for single dose primaquine for gametocyte clearance since the drug was developed in the 1960s (194). The trial was novel in being powered for both efficacy and safety outcomes. The original 0.75mg/kg dose was perceived as carrying an unacceptable risk of haemolysis in G6PD deficiency and the identification of a lower safe dose was key to enabling more widespread use. This opened the door to a new research agenda. The work incorporated trial design elements to evaluate transmission-blocking, rather than asexual efficacy and to assess safety in G6PD deficiency which have been adopted by subsequent investigators, and the field has expanded substantially to generate a battery of research to inform policy.

6.3 Limitations of the trial in the context of its application to diverse epidemiological settings

Due to resource limitations, the thesis did not incorporate mosquito feeding assays to assess the infectiousness of individuals to mosquitoes after primaquine treatment. Mosquito feeding studies demonstrate a more rapid action of primaquine than that determined using gametocyte clearance as an outcome (289). Primaquine efficacy was retained at lower doses when determined using membrane feeding assays compared to using gametocyte clearance as an outcome (256, 272). The use of the gene *pfs*25 as a molecular marker to detect gametocytes might mean that the efficacy of lower doses was underestimated. This is because the gene is detected in both viable and non-viable gametocytes. Hence, gametocytes rendered non-viable by primaquine, but not yet cleared by the spleen may have been detected in blood samples post treatment. The trial was carried out in an area with a level of malaria transmission above that at which elimination intervention might be initiated and the role that immune factors play in gametocyte clearance might differ across settings.

6.4 Policy recommendations and areas for future research

Coinciding with the use of this trial as a pre-read for the WHO Expert Review Group on The Safety and Effectiveness of Single Dose Primaquine as a *Plasmodium falciparum* gametocytocide, recommendations on the use of low dose primaquine were incorporated into WHO guidelines, setting in motion its adoption into policy globally.

However, despite clear evidence for its efficacy against gametocytes, this thesis highlights that several questions still remain to determine the best strategy for primaquine's use for optimal impact and safety. Furthermore, policy makers are experiencing substantial roadblocks in pursuit of its implementation.

Priorities include an exploration of primaquine's effectiveness for reducing community-level transmission, and investigation of the most high-impact and cost-effective strategy for its use, specifically as case-based treatment versus mass treatment. We need to further define primaquine's safety and operational studies are needed to embed strategies for safety surveillance that incorporates the most vulnerable members of a population. Epidemiological work aimed at clearly determining which individuals or groups sustain malaria transmission in endemic communities will determine where the parasite is most efficiently targeted with single low-dose primaquine.

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APPENDIX A

Trial Protocol

Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda

Funded by the Wellcome Trust

Principle investigator: Alice C. Eziefula, MBBS, MRCP, MRCPath

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Statement of Compliance

The study will be carried out in accordance with Good Clinical Practice (GCP) as required by the following:

- London School of Hygiene and Tropical Medicine Clinical Trials Sub-committee
- Makerere University School of Medicine Research Ethics Committee
- Ugandan National Drug Authority

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this study will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and ICH guidelines.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACT	artemisinin-based combination therapy
AUC	area under the curve
AE	adverse event
AL	artemether-lumefantrine
AS	artesunate
C_{max}	peak plasma drug concentration
CI	confidence interval
CRF	case record form
D0, D1, D2	day 0, Day 1, Day 2 of study medication administration
DSMB	data safety and monitoring board
EIR	epidemiological inoculation rate
G6PD	Glucose-6-phosphate dehydrogenase
GCP	good clinical practice
GCT	gametocyte clearance time
GMR	geometric mean ratio
Hb	haemoglobin
IDRC	Infectious Diseases Research Collaboration
IMCI	integrated management of childhood illnesses
ITN	insecticide treated net
IRB	institutional review board
HPLC	high performance liquid chromatography
LLIN	long-lasting insecticide treated net
LSHTM	London School of Hygiene and Tropical Medicine
PQ	primaquine
PQ1, PQ2	test doses of primaquine
PQ-R	reference dose of primaquine (0.75mg/kg)
QT-NASBA	real-time quantitative nucleic acid sequence-based amplification
SAE	serious adverse event
SD	standard deviation
SP	sulphadoxine-pyrimethamine
T_{max}	time to reach peak plasma drug concentration
UCSF	University of California, San Francisco
UMSP	Uganda Malaria Surveillance Project
UNCST	Uganda National Council of Science and Technology
WHO	World Health Organization

STUDY SUMMARY

Title	**Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria**
Study design	A randomized, double-blinded placebo-controlled clinical trial with 4 parallel arms
Participants and sample size	Individuals aged 1 year to 10 years with uncomplicated falciparum malaria Target sample size is 480 participants
Phase	III
Study site	Country: Uganda The study will be conducted at the Uganda Malaria Surveillance Project (UMSP) sentinel site in Walukuba, Jinja
Selection criteria	**Inclusion criteria:** 1. Age >/ 1 year and </10 years 2. Weight over 10kg 3. Fever >38 degrees C (tympanic) or history of fever in the last 24 hours 4. *P. falciparum* parasitaemia <500 000/µl 5. Normal G6PD enzyme function **Exclusion criteria:** 1. Enrolled in another study 2. Evidence of severe illness/ danger signs (Appendix A) 3. Known allergy to study medications 4. Haemoglobin< 8g/dL) 5. Started menstruation 6. Pregnancy or breastfeeding 7. Taken antimalarials within the last 2 days 8. Primaquine taken within the last 4 weeks 9. Blood transfusion within the last 90 days 10. Non-falciparum malaria co-infection
Study intervention	Participants receive AL on days 0-2 and are randomized to one of four treatment arms (below) on day 2. They are followed up for 28 days • Placebo • PQ1 (primaquine 0.1mg/kg) • PQ2 (primaquine 0.4mg/kg) • PQ-R (primaquine 0.75mg/kg)
General objective	To evaluate the efficacy and safety of different doses of primaquine administered with AL for the purpose of reducing *P. falciparum* gametocytes in the infected human host to prevent transmission of falciparum malaria the anopheles mosquito vector.
Specific objectives	1. To evaluate the efficacy of different doses of primaquine when administered with AL as measured by gametocyte prevalence and density 2. To evaluate the safety of different doses of primaquine when

<table>
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1. BACKGROUND

1.1 INTRODUCTION

The plasmodial parasite, malaria, infects an estimated 450 million people globally each year[1]. The majority of these infections occur in Sub-Saharan Africa where the predominate species, *Plasmodium falciparum*, is responsible for the greatest proportion of deaths worldwide due to malaria [2-3]. The five countries with the greatest number of malaria deaths in the world are Uganda, DRC, Nigeria, Ethiopia and Tanzania[1]. Aside from directly-attributable morbidity and mortality from severe malaria, malaria is responsible for a substantial all-cause mortality[4] and morbidity which is contributed to by anaemia[5], adverse pregnancy outcomes for mother and child[6] and long term sequelae of infection[7-9].

In Uganda, malaria transmission intensity is high and stable in most parts of the country. The national malaria control programme (MCP) supports the large-scale distribution of long-lasting insecticide-treated bed nets (LLINs), mosquito vector control with household indoor residual spraying of insecticide and intermittent preventive anti-malarial therapy in pregnancy. The other main strategy is effective diagnosis and case management.

Since 2004, the Ugandan national malaria treatment guidelines recommend artemisinin combination treatment (first line choice: artemether-lumefantrine [AL]) for uncomplicated malaria. This followed acknowledgment of the high level and widespread resistance to the previously recommended regimen of chloroquine plus sulphadoxine-pyrimethamine (SP).

Currently, the Ugandan national guidelines for treatment of severe malaria and of failure of first line treatment of uncomplicated malaria are under review given trial data on the efficacy of parenteral artemisinin treatment compared to the standard iv quinine.

Despite scaled-up control measures and support from international funding initiatives, the burden of malaria in Uganda has increased over the last decade and control remains a priority.

1.2 Global malaria control and elimination

A new global effort is underway to step up malaria control and push towards the elimination of malaria as a public health problem. This started in 2007 as a proposal by Bill and Melinda Gates and was supported by the WHO[10]. Since this declaration, some substantial successes have been achieved in shrinking the global distribution of malaria. In Africa, effective elimination programmes have been initiated in Zanzibar and South Africa. There are now 8 African countries with a commitment to malaria elimination (E8 Ministerial Resolution, Southern African Development Community 2009).

This call for elimination has created a drive for the development of new and innovative tools to reduce malaria transmission. One such tool is primaquine. It is a drug which can efficiently block the transmission of *Plasmodium falciparum* malaria from humans to mosquitoes.

Malaria is transmitted from mosquito vector to the human host by the injection of parasites from the mosquito mouthparts as it ingests a human blood meal. Onward transmission to the mosquito occurs when it feeds on an infected human host harbouring gametocytes, the sexual form of the parasite.

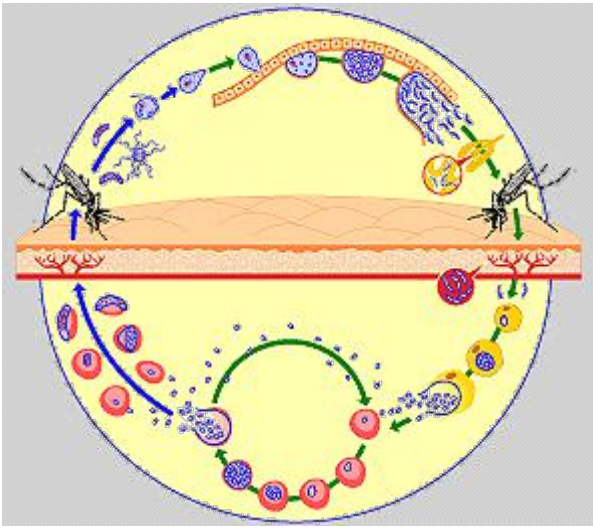


Figure 1 Malaria lifecycle (from TDR/ Wellcome Trust)

The WHO recommends the use of a single dose of primaquine as part of malaria elimination programmes:

"As the anti-gametocyte effects of artemisinins are incomplete, malaria elimination programmes require that artemisinin-based therapies be combined with primaquine to block transmission more effectively" (from Malaria Control and Elimination 2008, WHO publication).

1.3 PRIMAQUINE

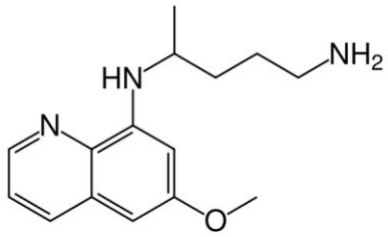


Figure 2 Primaquine- chemical structure

Primaquine is an old drug, developed in the 1940s and in widespread use since the 1960s. It was one of the first synthetic antimalarials to be developed. It belongs to the 8-aminoquinoline drug class. Other drugs in this class include Tafenoquine and Bulaquine, but these are not yet widely available. The 8-aminoquinolines are gametocytocidal, that is, they are active against the sexual forms of the *P. falciparum* malaria parasite, the gametocytes. These blood-borne sexual stages, although harmless to humans, are infectious to mosquitoes and are responsible for onward transmission of malaria from human to mosquito.

Primaquine is also effective against the sporozoites of *Plasmodium vivax* and *Plasmodium falciparum*, and against the hypnozoites of *Plasmodium vivax* and *Plasmodium ovale* but it has no effect on the blood stages of *Plasmodium falciparum.* Primaquine is most widely used for its effect against *P. vivax*

and *P. ovale* hypnozoites as anti-relapse therapy. For this purposes, it has been used for decades. In adults, the dosing of primaquine for PART is 30mg daily for two weeks.

Primaquine pharmacokinetics:

Pharmacokinetic data describes how a drug is managed (and metabolized) in different groups of individuals. Peak plasma concentration is within 1-4 hours[11-13] and the terminal half life is 4-6 hours[11].

Primaquine exhibits extensive tissue distribution[13-14]. About 75% of primaquine in plasma is bound to proteins and high concentrations occur in erythrocytes.

The parent drug is converted to its active metabolites in the liver. Less than 2% of the parent drug, primaquine is excreted in the urine within 24hrs of dosing[11]. Several metabolites of primaquine have been identified, but it is unclear which are responsible for the gametocytocidal action and which for its toxic effects. Carboxyprimaquine is the main metabolite [15]and its formation is cytochrome CYP450-dependent[16] . The 5-hydroxylated metabolite has been linked to both therapeutic efficacy and toxicity [17]. Other metabolites have been identified, but their function remains undetermined[18].

A high performance liquid chromatography (HPLC) method devised in 1984 [15][15][15][15][15][15][15][15][15][14][12][12]to detect primaquine with a sensitivity of 1ng/ml has been updated by Cuong [19].

Studies that provide pharmacokinetic data on primaquine have been conducted largely in Southeast Asia and Australasia. The majority of studies have been on adults. There is a lack of data on the pharmacokinetics and pharmacodynamics of primaquine in African children. Given that primaquine may be deployed in malaria endemic areas in Africa, this data is needed.

Side effects of primaquine:

Given its widespread use over the last fifty years, there is extensive experience with regards the safety and side effects of primaquine. The main side effects are as follows:

- Gastro-intestinal symptoms if not given with food
- Methaemoglobinaemia
- Transient haemolysis in individuals with a predisposition such as G6PD deficiency. The haemolysis is mostly in aged erythrocytes (red blood cells). Therefore, the retiulocytosis (proliferation of young red blood cells) in acute malaria affords some protection, as the population of red ells is relatively younger.

The side effects are **dose-related**. Therefore, at lower doses the side effects are expected to be less or insignificant. It is common practice to give individuals with G6PD deficiency a lower dose of 0.75mg/kg once weekly) as treatment for *P. vivax* (WHO Malaria Treatment Guidelines 2010).

1.4 G6PD (GLUCOSE-6-PHOSPHATE DEHYDROGENASE) DEFICIENCY

The glucose-6-phosphate dehydrogenase (G6PD) genetic polymorphism was discovered through the observation that certain individuals had the tendency to haemolyse (undergo destruction of red blood cells) when primaquine was administered[20]. Subsequently, other triggers have been discovered that promote haemolysis in individuals with G6PD deficiency.

The G6PD polymorphism is conserved in malaria-endemic regions and this has led to speculation that alleles coding for deficiency of the enzyme afford protection against *Falciparum* malaria infection or against death from malaria.

G6PD enzyme function varies widely in different regions across the globe due to the polymorphism.

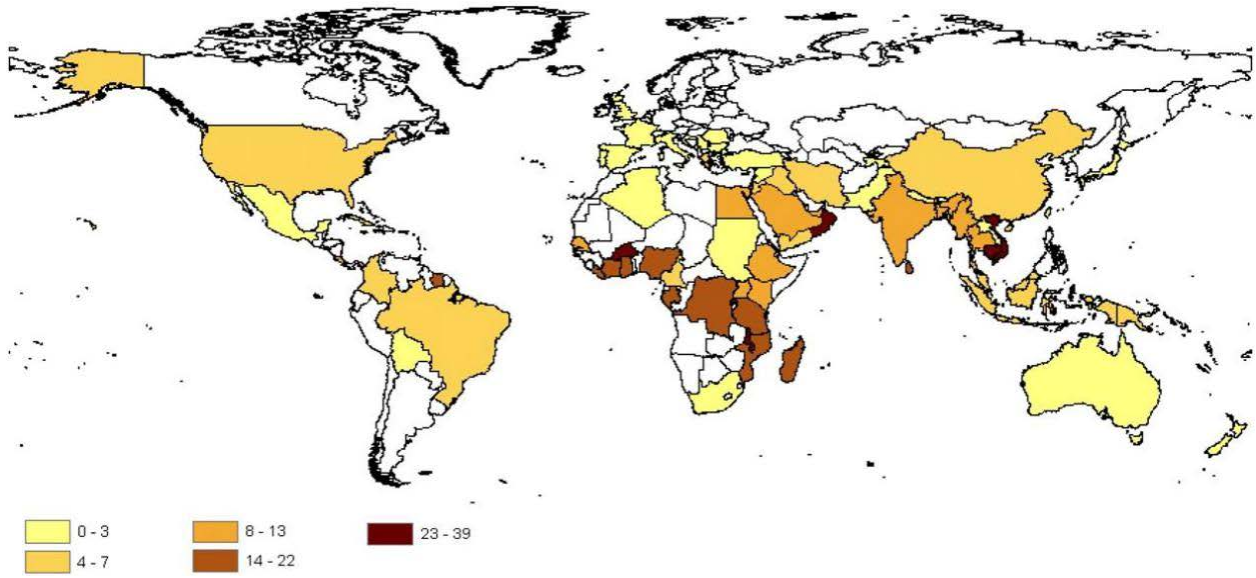


Figure 3 Crude average percentage G6PD prevalence from Nkhoma *et al.* 2009[21]

The gene codes for an enzyme which catalyses the first step of the pentose phosphate pathway for glucose metabolism in red blood cells. The range of mutant alleles (over 140 have been characterized) result in varying degrees of deficiency of this enzyme. G6PD enzyme deficiency causes a reduction in this enzyme function. This leaves red cells with lower amounts of NADPH (reduced nicotinamide adenine dinucleotide phosphate) with the result that they are susceptible to oxidative stress. Subsequent oxidative stress can lead to haemolysis. Primaquine exposure leads to transient, dose-dependent intravascular haemolysis in individuals carrying the mutant allele. The severity of the haemolysis depends on the degree of enzyme deficiency.

The most common G6PD variant in Africa is the A- variant. This codes for a relatively mild deficiency of the enzyme. In contrast, some Southeast Asian and Mediterranean variants code for severe deficiency, whereby one single dose of a trigger compound, such as primaquine can provoke a severe haemolysis, requiring treatment (typically with blood transfusion and supportive measures).

G6PD deficiency is an X-linked trait, meaning that the male hemizygote (males who carry the gene on their single X chromosome) has full expression of the trait. The prevalence of a given allele in a population is therefore commonly described as the percentage of males carrying the gene. Because it is carried on the X chromosome, females can have a variety of levels of gene expression. This is because

they exhibit "lyonisation". Lyonisation is the tendency to randomly inactivate one of the X chromosomes in every cell. Therefore, randomly, some females, although they carry the deficient allele, will have normal enzyme function, whilst others may have varying levels of deficiency. Therefore, the deficiency is less expressed in females at the population level.

The genotype (genetic code) can code for different phenotypes (actual level of enzyme function), depending on the sex of the individual and the allele they carry (which variant they carry). Therefore, it is important to distinguish whether G6PD deficiency is being measured as a person's genotype or as their enzyme function.

1.4.1 G6PD deficiency in Uganda.

In an urban household survey in Kampala, 16% of male children and 10% of female children had reduced G6PD enzyme function [22] and reduced enzyme function was associated with reduced risk of malaria parasitaemia.

The most common G6PD variant in Uganda is the A- variant. This comprises alleles from the A variant (G376A) and the G202A mutation. In up to 5% of A- variants, mutations occur at nucleotides 680 and 968 in the gene coding for G6PD.The A- G6PD variant has up to 80% enzyme function compared to wild type. This variant is associated with mild haemolysis in the presence of stimuli such as primaquine.

Table 1 G6PD variants, geography and broad risk of haemolysis

G6PD variant	Geographic region	Risk/ severity of haemolysis
B (Wild type)	Worldwide	None
A	Africa	Mild
A-	Africa South America	Mild-moderate
Mediterranean	Middle East, Europe, South Asia	Severe
Viangchan	Southeast Asia Australasia	Mild-moderate-severe
Mahidol		
Vanua Lava		
Canton		
Anant		
Kaiping		
Seattle	Mediterranean, Western Europe, North Africa	Mild-moderate
Union	Mediterranean, Western Europe, North Africa, China, Pacific Islands	Moderate-severe

1.5 A NEW INDICATION FOR AN OLD DRUG: Primaquine for transmission-blocking

The WHO first recommended primaquine for transmission-blocking in the 1970s. It was not until recently that this application has received more attention in the recent WHO recommendations in 2008 and 2010. Despite this history, there is less widespread familiarity with primaquine as a transmission-blocking drug compared to the experience with its use in radial cure of *P. vivax* malaria.

- ***Are there alternatives to primaquine for transmission-blocking?***

Artemisinin derivatives have some gametocytocidal action, being effective against developing gametocytes (stages 1 to 3 gametocytes). This may explain the reduction in malaria transmission in settings where their use is well-established [23-24]. However, following artemisinin combination therapy, microscopic and sub-microscopic (using molecular techniques) gametocytaemia is still detectable and individuals are still infectious to mosquitoes, i.e. transmission to mosquitoes can still occur[25]. The only drugs available which are highly effective against mature gametocytes (stages 4 to 5) are the 8-aminoquinolines; primaquine being the least expensive and most widely available.

- ***How is it given?***

The dose for transmission-blocking is much lower than the dose for radical cure of *P. vivax*. Instead of a 14 day course, it is one single dose of 0.75mg/kg.

- ***What is the evidence?***

The following studies provide data on the efficacy and safety of 0.75mg/kg single dose primaquine for transmission-blocking.

Data from Africa:

A recent study conducted in Tanzania[26] in asymptomatic parasitized children demonstrated a dramatic reduction of gametocyte circulation time with primaquine treatment from 28.6 days in the absence of primaquine (with ACT alone) to 6.3 days with primaquine.

Primaquine reduced gametocytaemia significantly at days 4, 7, 14 and 28 post treatment in a Tanzanian study[27] comparing ACT with or without primaquine in children with uncomplicated clinical malaria. Here, the prevalence of gametocytes on day 14 after treatment was reduced from 62.7% to 3.9%.

Shekalaghe *et al[27]* demonstrated that the addition of a single dose of primaquine to ACT in Tanzanian children aged 3 to 15 years with uncomplicated malaria and unknown G6PD status at baseline caused a maximal mean drop in haemoglobin on day 7 post treatment initiation (5 days after primaquine, which was given on day 2 after ACT treatment initiation). Mean haemoglobin fell by 5.2% from enrolment value. The greatest fall in haemoglobin was noted in the children with G6PD deficiency. However the study was not powered to detect a difference in outcomes by G6PD variant. By 28 days, haemoglobin values in all children were no longer significantly different to enrolment values. None of the children required transfusion or had symptomatic anaemia.

When primaquine was given to Tanzanian children with asymptomatic malaria infection in later study [28], the mean change in haemoglobin at day 7 post treatment was -0.58g/dL and -2.5g/dL in the children with G6PD A- variant genotype. One child developed severe anaemia, but this child was not in the G6PD A- variant group, having genotype A and recovered with haematinics. No children required blood transfusion.

A study conducted in Sudan[29] showed no significant difference in gametocyte prevalence on day 7 or day 14 post treatment with or without primaquine in individuals with asymptomatic infection. This study was conducted in the dry season in an area with high seasonality for malaria infection. This highlights the need for more data to define the efficacy of primaquine in different transmission settings. No serious or severe adverse events were reported.

Data from elsewhere:

In Thailand[30], patients presenting with uncomplicated malaria in Bangkok had reduced gametocyte clearance times when primaquine was added to all drug combinations. Primaquine reduced gametocyte clearance with an odds ratio of 0.42 (0.20 to 0.83); *P* =0.009.

In a recent study in Burma[31], 808 participants were randomized to receive ACT plus primaquine or ACT alone. Gametocyte carriage was substantially reduced by the addition of primaquine (rate ratio 11·9 (95% CI 7·4–20·5; *P* =0.0001). There was an overall increase in haemoglobin during follow up in both the primaquine and the non-primaquine arms, but the increase was smaller in the primaquine group (0.75 g/dL *vs* 1.04 g/dL; =0.036; mean difference 0.295 g/dL; 95% CI 0.199–0.570). There was no severe anaemia. This study provided detailed adverse events analysis and there were no severe adverse events. The only adverse event attributable to primaquine was abdominal pain. This is a known side effect and is reduced by administration with food[19].

In Colombia (2010)[32], investigators found a disappearance of gametocytes one week earlier when PQ was added to an artemisinin-containing regimen.

In Cambodia, 3653 individuals received 0.75mg/kg primaquine (without G6PD screening) every ten days in a mass drug administration programme and there were no major adverse events[33].

OUTSTANDING QUESTIONS ON THE USE OF PRIMAQUINE AS A TRANSMISSION-BLOCKER

Aside from basic information on the safety and efficacy of primaquine as a transmission-blocker, several important questions remain to be answered when it comes to considering how primaquine should be used:

- **What is the optimal dose of primaquine?**

The dose of 0.75mg/kg as a single dose dates back to the 1940s. A single dose of the daily dose used for *P. vivax* was found to clear *P. falciparum* gametocytes. Adequate dose-finding data are lacking and are now needed urgently.

Studies using lower doses than 0.75mg/kg for *P. falciparum* transmission-blocking

Two studies in Thailand have demonstrated that lower doses of primaquine had indistinguishable efficacy to higher doses. Bunnag [34](1980) compared the effect of 15mg daily for 5 days, 30mg single dose and 45mg single dose in Thai adults and found no significant difference in gametocyte clearance between doses. Pukrittayakamee [30](2004) compared 0.25mg/kg and 0.5mg/kg primaquine in adults and found both to have shorter gametocyte clearance times (GCT) than non-primaquine-containing regimens, with no significant difference in outcomes between the two doses of primaquine. Clearly, there is a requirement for more detailed dose-response data.

The mass drug administration programme in Cambodia[33] used a 9mg stat dose of primaquine (approximately 0.15mg/kg) every ten days, with a significant reduction in microscopic gametocyte carriage from 13.1% to 0.8% after 3 years.

- **On what day of treatment should primaquine be added?**

If primaquine is to be given in clinical case treatment or mass treatment initiatives, it is much cheaper and more reliable to give it at the same day as the partner asexual treatment so that individuals do not need to return to the health facility or be reached on days after the first point of contact. However, often primaquine is given *after* the start of treatment (e.g. on day 3 of a 3 day ACT course) in order to avoid exacerbating the nadir in haemoglobin associated with clinical malaria. Based on a gametocyte half life of 4-6 days, some authors suggest giving primaquine on day 7 or 8 to capture maturing gametocytes which develop in the first few days of treatment[35]. Few studies have examined the efficacy associated with the timing of primaquine treatment. Lederman[36] found a shorter GCT when primaquine was given on day 2 rather than day 0, but this was not significant. Research is required to identify the optimal timing of primaquine administration for safety and efficacy.

- **Where should primaquine be used?**

  Much of the pharmacokinetic data on primaquine is available from studies conducted in Southeast Asia. In these populations, the genetic susceptibility to primaquine sensitivity (G6PD deficiency) is very different to that in Africa. As elimination programmes move their focus to Africa, it is important that quality data are available for its use in Africa.

- **When should primaquine be used?**

  At what stage of malaria control should primaquine be introduced? So far, primaquine has been introduced mainly in countries on the brink of elimination (in Africa, examples are Botswana, Madagascar, South Africa and Zanzibar), but as malaria control efforts increase in higher transmission countries, it is likely there will be opportunities to reduce transmission with primaquine in sub-regions with lower malaria endemicity.

- **How should primaquine be used operationally?**

  Data are required to clarify how best primaquine should be used to block transmission on a population level. Should it be given as additional treatment to clinical cases of malaria, or should it be given as part of a mass treatment and screening initiative, to people with asymptomatic infections? As such, quality information on the safety and efficacy of primaquine is needed.

The WHO recommendation is rapidly coming into policy. Primaquine is being introduced or considered in many settings. It may be a very useful tool to reduce malaria transmission, but urgently, we need data to inform policy makers on the appropriate and safe use of the drug.

Of the few studies which have assessed primaquine efficacy for transmission-blocking, none are adequately powered and randomized to assess safety outcomes as well. There is a lack of quality data to inform policy makers on the safety of primaquine for transmission-blocking.

We have chosen to investigate the most pressing issue, the effective and safe dosing of primaquine for transmission-blocking.

We hypothesize that lower doses of primaquine may be effective at transmission-blocking, but have a much better safety profile.

This is particularly important in regions where G6PD deficiency is prevalent (See Section +++++G6PD deficiency)

2.0 RATIONALE

Malaria is a major public health problem. Every year, approximately one million people die from malaria and the majority of these are children aged less than five years. Current malaria control efforts are inadequate, despite a new drive for malaria elimination since 2007[10].

In 2008, the WHO recommended that, to block transmission of falciparum malaria, a single dose of primaquine should be added to ACTs in malaria control and eradication programmes (WHO "Malaria Control and Elimination", 2008). Primaquine is a member of the 8-aminoquinoline drug class. This is the only drug class with activity against the mature gametocytes of *P. falciparum*, the form of the parasite which is responsible for onward transmission from humans to mosquitoes. Primaquine is the most widely-available drug in this class and its cost is low. We have less experience with other drugs in this class.

Following the WHO recommendations, primaquine is rapidly coming into use as a transmission-blocker in malaria control programmes and it is estimated that millions of people stand to receive doses for this purpose annually[37]. Hence, high-quality regional data on primaquine's safety and efficacy are required urgently.

A single dose of 0.75mg/kg primaquine base is recommended for transmission-blocking. However the optimal dose for safety and efficacy has never been evaluated. Dose-finding data is important because primaquine has a dose-dependent risk of causing haemolysis (destruction of blood cells) in pre-disposed individuals, such as those with G6PD deficiency. G6PD deficiency is most prevalent in malaria-endemic areas. Therefore, it is essential that data on primaquine's safety is available in such areas.

Quality data are required to establish the safety of a single dose of primaquine in African children. Few studies have looked at the efficacy and safety of lower doses of primaquine than that recommended by the WHO for transmission-blocking. Those that have looked have found that lower doses still significantly reduce transmission/ gametocyte prevalence compared to placebo. No studies have compared the WHO dose to lower doses. A comparison of lower doses against the WHO dose and controlled against placebo is required because of the dose-dependent side-effects of primaquine. Pharmacokinetic data are also needed. No studies have documented the pharmacokinetics of primaquine in African children.

We hypothesise that lower doses of primaquine have a substantially lower risk of, or an absence of adverse effects compared to the WHO-recommended dose, but retain the transmission-blocking efficacy.

We propose to test this hypothesis in a four-arm clinical trial with a non-inferiority design to evaluate the efficacy and a superiority design to evaluate the safety of the WHO dose (0.75mg/kg) and lower doses of primaquine for clearance of *P. falciparum* gametocytes in children in Uganda. The study will include a pharmacokinetic analysis.

3.0 STUDY OBJECTIVES

3.1 GENERAL OBJECTIVE

To evaluate the efficacy and safety of different doses of primaquine administered with AL for the purpose of reducing *P. falciparum* gametocytes in the infected human host to prevent transmission of falciparum malaria to the anopheles mosquito vector.

3.2 SPECIFIC OBJECTIVES

1. To evaluate the efficacy of different doses of primaquine when administered with AL as measured by gametocyte prevalence and density

2. To evaluate the safety of different doses of primaquine when administered with AL as measured by change in mean haemoglobin, prevalence of severe anaemia (Hb <5g/dL), and evidence of black urine (haemoglobinuria; dipstick positive)

3. To assess the safety of different doses of primaquine when administered with AL as measured by prevalence/ incidence of adverse events and tolerability

4. To obtain basic pharmacokinetic parameters for primaquine in the study population

4.0 STUDY DESIGN/ METHODS

4.1 STUDY DESIGN OVERVIEW

The study is a randomized placebo-controlled trial with four parallel arms. A total of 500 individuals will be enrolled. Participants will be recruited from the Health Centre IV in Walukuba, Jinja if malaria is suspected, that is they have a history of fever at presentation and a positive malaria thick film.

Prior to undergoing any study procedures, individuals will be screened by the study clinicians for eligibility to enter the study. If they satisfy initial criteria, individuals will be invited to give informed consent to participate in the clinical trial. Children over 8 years of age will be invited to give assent to participation in the clinical trial. Consenting participants will then undergo clinical and laboratory screening. If they satisfy the study selection criteria, they will be enrolled in the trial. A small minority may be excluded from the trial after day 0, when final laboratory screening results become available. If individuals do not satisfy selection criteria, their malaria infection will be managed by the local clinic staff.

All enrolled individuals will receive a full three-day course of AL, and will be randomized to receive a dose of primaquine or placebo with their last dose of AL on day 2. All doses of AL and PQ will be directly observed. Sampling will be as follows: All individuals have finger prick blood samples on days 0, 1, 2, 3, 7, 10, 14, 21 and 28 for malaria parasites (asexual and sexual), haemoglobin (using Hemocue®) and into an EDTA tube for gametocyte molecular analysis. In cases where there is insufficient blood via finger prick, a venopuncture sample may be obtained. On each day of follow up, there will be an assessment by a clinician and an assessment for adverse events. Participants will be reimbursed for travel to and from

the clinic for all scheduled and non-scheduled visits during the time they are enrolled in the study. On day 1, every 4th child enrolled will be invited to consent for pharmacokinetic analysis on days 2-4. During the 28 days of follow up, all participants will be encouraged to attend the clinic for any medical concerns and the cost of travel to the clinic will be reimbursed.

4.1.1 RECRUITMENT PROCEDURES OVERVIEW

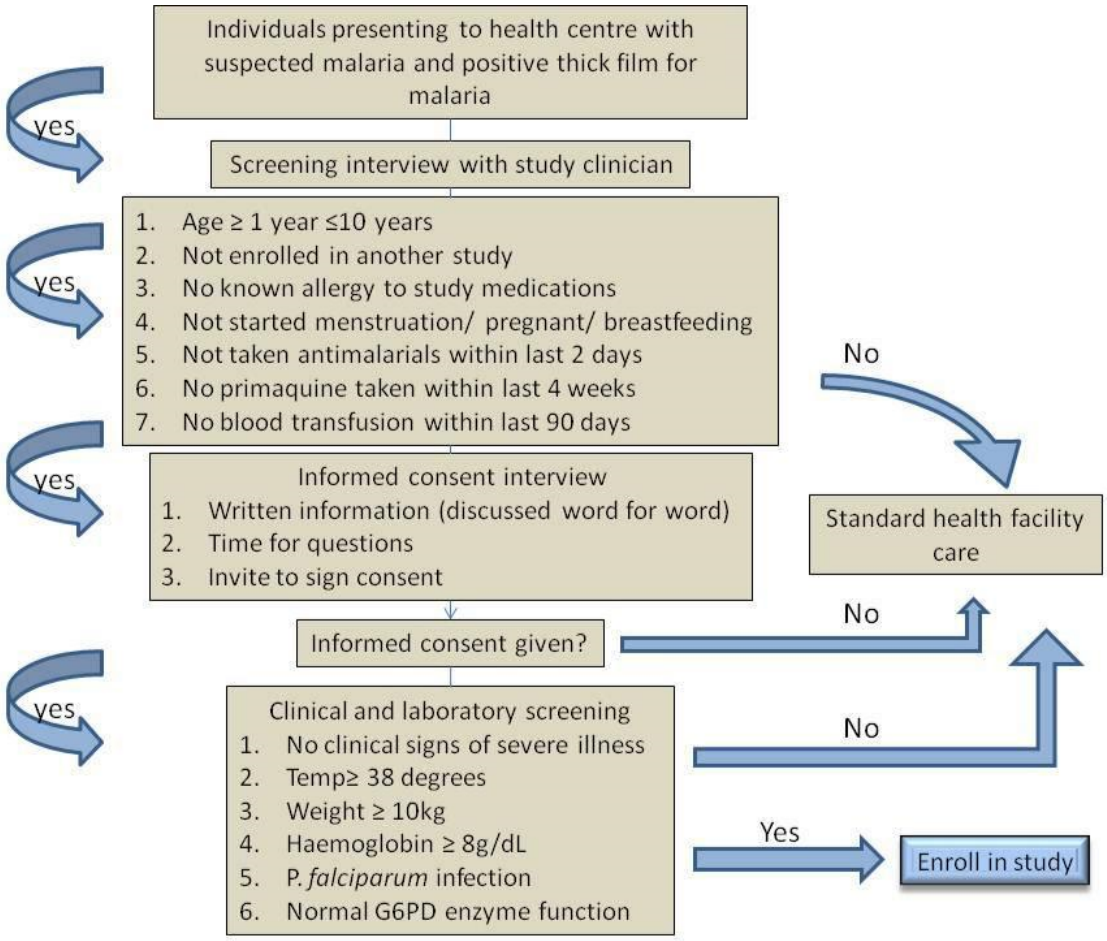


Figure 4 Recruitment procedures

4.1.2 TREATMENT AND FOLLOW UP OVERVIEW

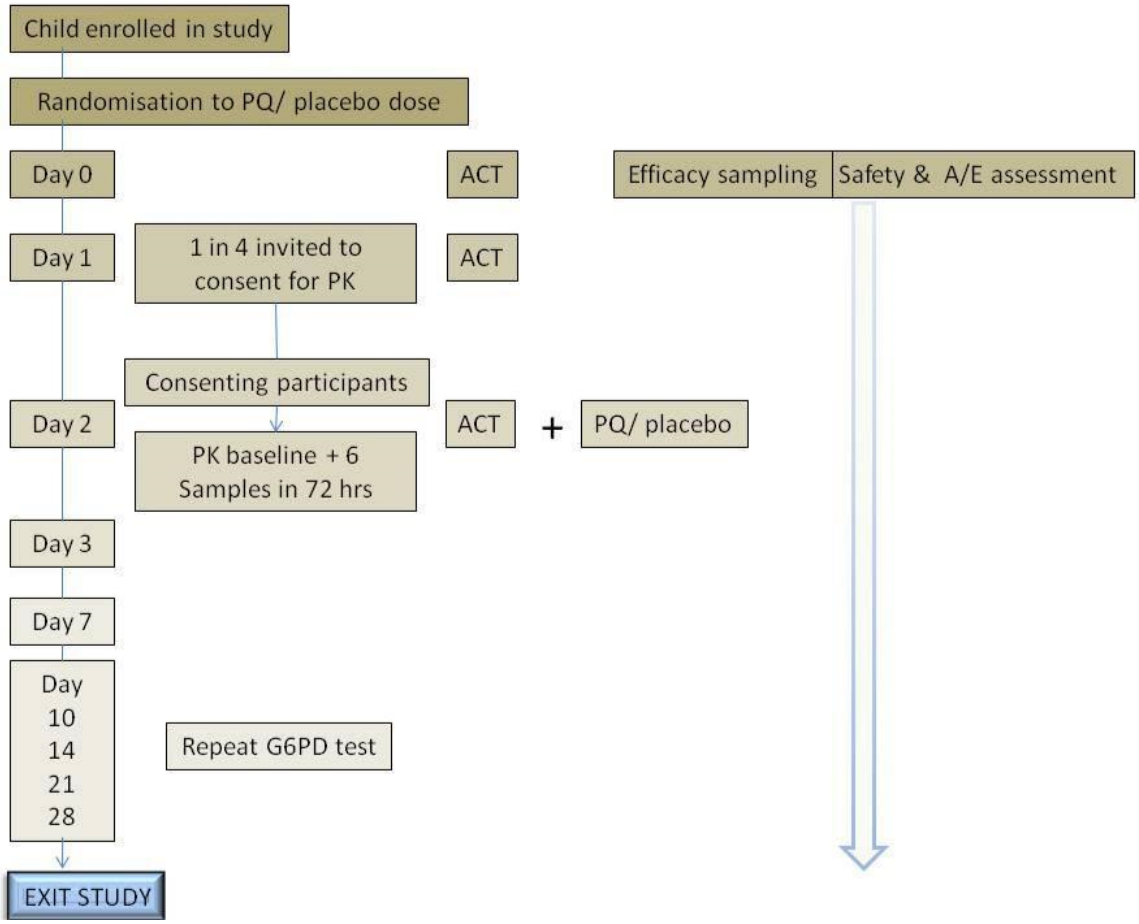


Figure 5 Treatment and follow up

4.2 OUTCOME MEASURES

These are summarized in table 2

Table 2 Outcome measures

		OUTCOME MEASURE	DESCRIPTION
EFFICACY			
	PRIMARY	**Mean number of days to gametocyte clearance (gametocyte clearance time, GCT)**	**Mean number of days per treatment arm for gametocytes to become undetectable using sub-microscopic molecular testing methods (QT-NASBA). -Re-appearance of gametocytes after day 14 will be considered re-infection and excluded.**
	SECONDARY	Mean (+/- SD) area under the curve of gametocyte density per day during 14 days of follow-up	Total number of gametocytes (measured by QT-NASBA) seen over follow up, averaged per day of follow up (days 0-14)
		Point prevalence of gametocytes on days 7, 10 and 14	Mean number of gametocytes (measured by QT-NASBA) per treatment arm on days 7, 10 and 14
		Proportion (%) of participants with gametocytes on each day of follow up	For each treatment arm, percentage of participants with gametocytes (measured by QT-NASBA) on each day of follow up from days 0-14.
SAFETY			
	PRIMARY	**Mean (+/- SD) maximal fall (+/ or -) in Hb (g/dL) from enrollment to day 28 of follow-up**	**Mean maximal greatest negative difference in Hb (measured by Hemocue®) from enrollment value per treatment arm over 28 days follow up**
	SECONDARY	Follow-up day of Hb nadir	Mean day of follow up (day 0-28) per treatment arm of lowest Hb measurement (by Hemocue®)
		Maximal percentage fall in Hb level compared to enrolment value	Size of maximal Hb drop (by Hemocue ®) during follow up (day 0-28) from enrollment value, divided by enrollment value, *100
		% participants with Hb < 5g/Dl during follow up	Percentage(number) per treatment arm during days 0-28

	Requirement for blood transfusion	Percentage (number) of children receiving blood transfusion per treatment arm during days 0-28
	Evidence of black urine	Percentage (number) of children with documented black/ dark urine with urine dipstick positive for Hb per treatment arm during days 0-28
	Incidence of serious adverse events by sign, symptom, laboratory parameter and relationship to taking study drug	Percentage (number) per treatment arm during days 0-28
	Incidence of gastrointestinal symptoms after taking study drug	Percentage (number) per treatment arm during days 2-7

4.3 SELECTION CRITERIA

Complete selection criteria are listed as follows:

Inclusion criteria:

1. Age >/ 1 year and </10 years
2. Weight over 10kg
3. Fever >38 degrees C (tympanic) or history of fever in the last 24 hours
4. *P. falciparum* parasitaemia <500 000/µl
5. Normal G6PD enzyme function

Exclusion criteria:

1. Enrolled in another study
2. Evidence of severe illness/ danger signs (Appendix A)
3. Known allergy to study medications
4. Haemoglobin< 8g/dL)
5. Started menstruation
6. Pregnancy or breastfeeding
7. Antimalarials taken within the last 2 days
8. Primaquine taken within the last 4 weeks
9. Blood transfusion within the last 90 days
10. Non-falciparum malaria co-infection

4.4 STUDY SITE

The study will be conducted at Walukuba Health Centre IV in Walukuba, Jinja. The EIR (entomological infective rate; the approximate number of infective bites per person per year) in Walukuba is estimated to be 7 (Okello 2006 ASTMH).

Walukuba Health Centre IV is in a peri-urban environment. The catchment area is up to 10km from the health centre, which incorporates some rural areas. Some individuals in the catchment area live on islands in Lake Victoria. From Walukuba Health Centre IV, there is good road access to the Jinja District Hospital where inpatient facilities and regional specialist paediatric services are available.

The health centre has been used as a research site in the past and as a result, there is a good link between laboratory and clinic. The health centre is a sentinel site for the Uganda Malaria Surveillance Project (UMSP). Consequently, malaria diagnostic services are highly efficient. Much of the infrastructure required for clinical research in Walukuba has been established by UMSP. The proximity to Kampala is an advantage, with respect to research facilities and specialist medical services available at Mulago National Referral Hospital.

4.5 PARTICIPANT SELECTION AND ENROLLMENT

6.5.1 RECRUITMENT (Triage and blood slide)

Study subjects will be recruited from the outpatient department of Walakuba Health Center IV in Jinja. As per usual practice in the health centre, all patients who present to the outpatient department will be seen by the health centre health workers for triage. Those with symptoms suggestive of malaria will be referred to the laboratory for a screening thick blood smear. Screening blood smear slides will be read and counted by the outpatient laboratory technicians. Any patient with a positive screening thick smear will be referred to our clinic for further evaluation.

4.5.2 INITIAL SCREENING BY STUDY CLINICIAN.

Upon referral to the study clinic, a standardized screening interview will be conducted by study physicians. This interview will go through the initial screening selection criteria (below). If the patient fulfills the initial screening criteria, the informed consent process will be initiated prior to examining the participant and performing any laboratory tests. All patients who are excluded from study enrollment will be referred back to the standard outpatient clinic for treatment of their malaria infection and other appropriate care.

4.5.2.1 INITIAL SCREENING CRITERIA

Inclusion criteria:

1. Age >/ 1 year and </10 years

Exclusion criteria:

1. Enrolled in another study
2. Chronic severe illness
3. Primaquine taken within the last 4 weeks
4. Known allergy to study medications
5. Started menstruation
6. Pregnant or breastfeeding
7. Blood transfusion within the last 90 days

4.5.3 INFORMED CONSENT PROCESS

The process for obtaining informed consent involves 4 steps:

1. Study introduced verbally (discussion)
2. Written information provided and read through word for word
3. Time for asking questions
4. Written consent given by participant/ guardian

Study physicians will seek formal consent in the clinic. The consent form (Appendix C) comprises written information on the study and a section for declaration of consent and signature. After introducing the study, the information will be read to the parent/ guardian word for word by the study staff. The information will be available in the native language of the parents/ guardian (Luganda, Lusoga, Swahili, or English). A translator will be used if necessary. The information provided will be a full description of the study with details of the implications for the individual participant, and the constraints of the protocol, the known side effects and any risks involved in taking part. It will be clearly stated that the participant is free to withdraw from the study at any time for any reason without prejudice to future care, and with no obligation to give the reason for withdrawal.

Adequate time will be allowed for the participant or parent/ guardian to consider the information and to ask questions.

They will then be invited to sign the written consent form adjoining the written study information and approved by the IRBs for their child to participate in a research study and a second consent for the future use of biological specimens obtained during the course of the study (Appendix D). If the parent or guardian is unable to read or write, their fingerprint will be used in substitute for a signature, and a signature from an impartial witness to the informed consent discussion will be obtained. Two copies of the consent form must be signed. The parent/ guardian/ impartial witness will sign/ fingerprint one copy for the study staff and one copy to keep for themselves.

If the child is 8 years of older, they will be invited to give written assent to participate in the study. The assent form (Appendix E) will be read through word for word and a witness signature will be requested.

Following the informed consent discussion, parents (or guardians) will be given their copy of the form to keep which includes the study information, the signed consent form and contact names and telephone numbers to use if they have further questions regarding the study or follow up procedures. If assent is obtained, the participant will keep their signed copy.

4.5.4 CLINICAL SCREENING

Following the consent process, further screening will be conducted to determine whether the individual is eligible to participate in the trial, according to the study selection criteria. The clinician will take a brief, relevant history. If the clinician has any concerns that a female child may have undergone puberty and could be at risk of pregnancy, despite a history that she has not started menstruating (section

4.5.2.1) then the clinician will recommend a pregnancy test. If the participant/ guardian declines a pregnancy test, then the child will be excluded. If a child is pregnant, she will be excluded from the study and referred for antenatal care and counseling. A clinical examination will be conducted to exclude signs of severe illness.

4.5.4.1 CLINICAL SELECTION CRITERIA

Inclusion criteria:

1. Weight over 10kg

2. Fever >38 degrees C (tympanic) or history of fever in the last 24 hours

Exclusion criteria:

1. Evidence of severe illness/ danger signs (Appendix A)

4.5.5 LABORATORY SCREENING

If the clinical selection criteria are met, patients will go to the study laboratory and have a single venous blood sample in EDTA. This will be for baseline laboratory tests including haemoglobin (Hemocue®), thick and thin blood smears (Giemsa-stained) and G6PD enzyme level (fluorescent spot test), molecular gametocyte assay (QT-NASBA) and filter paper samples. The participant will return to the study clinician with the haemoglobin results.

The clinician will assess whether the haemoglobin criteria are satisfied:

1. Haemoglobin ≥8g/dL

If haemoglobin criteria are satisfied, then the participant will be enrolled in the trial and they will be treated promptly with anti-malarial medication (AL).

Four further inclusion criteria (below) will be assessed during the first 24 hours after enrollment by a study laboratory technologist who will be blinded to treatment group assignments. Results of the Giemsa-stained thick and thin blood smears will not be available until after the patients have been treated and discharged from the clinic. Thus, although it is unlikely, it could be possible for a patient to be excluded from the study after enrollment and AL treatment if these criteria are not met**.** Patients who are excluded on Day 1 for the following criteria will be treated and followed appropriately in the study clinic.

1. Successful phlebotomy
2. P. falciparum mono-infection
3. P. falciparum parasitaemia less than 500 000/µl
4. Normal G6PD enzyme level

G6PD enzyme level is considered normal if there is fluorescence with the fluorescent spot test[38].

4.5.6 ENROLLMENT

All patients who have given consent and satisfy the screening criteria will be seen by a study clinician for enrollment (Appendix G).

They will be assigned a study number.

All participants will be given a study clinic follow up appointment card. This will give their follow up dates and study ID number.

The enrollment procedures are listed below:

4.5.6.1 PATIENT HISTORY

- History of presenting complaint. Include documentation of history of fever.

- Relevant past medical history
- Drug history including allergies/ adverse reactions if known.
- Demographics. Age and sex, preferred language. (Note: GPS readings of the household are to be taken by the fieldworker on bringing the patient home.)

4.5.6.2 PHYSICAL EXAMINATION

Examination of respiratory, cardiovascular, abdominal, nervous and musculoskeletal systems, ear, nose and throat, skin and nutritional status.

4.5.6.3 LABORATORY INVESTIGATIONS

Baseline analysis

The following additional baseline tests are assessed from the blood sample taken by the phlebotomist in the clinic laboratory at screening:

- malaria thick and thin blood smear (Giemsa-stained in clinic laboratory)
- haemoglobin (Hemocue®)
- EDTA (samples for quantification of gametocytes using molecular method [quantitative nucleic acid sequence-based amplification, QT-NASBA] and filter paper samples for future use)
- quantitative G6PD enzyme function (ELISA)

	SCREENING	ENROLLMENT
Laboratory test	Hospital triage finger prick	
	Malaria thick blood smear	na
	Study clinic EDTA venous sample	
	Malaria thick and thin blood smear (Giemsa-stained)	QT-NASBA buffer and filter paper samples
	Haemoglobin (Hemocue®)	Quantitative G6PD enzyme function (ELISA) on filter paper
	Qualitative G6PD enzyme function (Fluorescent spot test)	

Table 3 Summary of laboratory tests in screening and enrollment

4.6 STUDY INTERVENTION

4.6.1 RANDOMIZATION

After enrollment, participants will be assigned to a treatment group using a randomized method stratified by sex. The responsible study staff will select sequential opaque envelopes (from either the male or female pile). Each envelope contains a pre-determined treatment assignment code. The study nurse will bring the envelope to the study pharmacist.

4.6.2 ALLOCATION CONCEALMENT

The study pharmacist will possess the assignment code breaker and will dispense the relevant treatment for days 0-2. The treatment assignment code corresponds to a PQ dose to be given on day 2: P0 (placebo), P1-3 (variable dose primaquine) and the study pharmacist has access to the code but the study nurses and clinicians do not.

Having selected an opaque envelope for the child, the study nurse will bring the envelope to the study pharmacist. The study pharmacist will open the envelope, document the treatment assignment code and the participant's study number on the treatment assignment log, calculate the correct dose of primaquine/ placebo in milligrams and document the number of millilitres of primaquine/ placebo solution that are required. The treatment assignment code and the dose to be given will not be documented on the CRF or provided to the study nurse.

4.6.3 BLINDING

The study pharmacist will be the only member of the clinic team not blinded to the treatment groups. The study pharmacist will not have patient contact and will not be involved in assessing patients or assigning outcomes.

The study site staff who are administering drugs assessing patients and processing laboratory samples will not have access to the randomization code breaker.

The participant will not be informed of the PQ dose to be administered

The primaquine dose will be placebo-controlled. All participants will receive a second treatment on day 2. Placebo will be as indistinguishable as possible from PQ, both being dissolved tablets in solution and of the same volume.

4.6.4 PROCEDURES FOR RANDOMIZATION, ALLOCATION CONCEALMENT AND BLINDING

The randomization and treatment allocation process is summarised below:

- Upon enrollment, nurse selects next opaque envelope according to the child's gender. The envelope contains the participant's allocation code. The allocation code corresponds to one of the four treatment arms
- Study nurse labels envelope with the participant's study number and weight
- Study nurse presents envelope to pharmacist to request treatment. The pharmacist opens the envelope and documents the participant's study number and allocation code on the treatment assignment log
- Study pharmacist uses participant's weight and assignment code to calculate the correct primaquine dose or the equivalent dose of placebo to be given on day 2 (Appendix I)
- Study pharmacist logs this dose in the treatment assignment log together with the participant's treatment allocation code and study number. There is one treatment assignment log for each

treatment arm (PQ1, PQ2, PQ3 and placebo). The study pharmacist calculates and documents the number of millilitres of PQ/ placebo solution that will need to be given on day 2

- The study pharmacist dispenses the six AL doses when the nurses request (morning and evening of days 0-2

- The study pharmacist labels the AL treatment assignment log form with the participant study number, treatment assignment code and AL batch number

- For all treatments (PQ/ placebo and AL), the study nurse will document that the treatment has been given, the number of tablets/ millilitres of drug given and whether or not the dose was vomited or repeated

4.6.5 TREATMENT ADMINISTRATION: PROCEDURES

All treatments will be directly observed. A small snack will be administered prior to both AL and primaquine administration.
Details of the study drugs are summarized in table 4 below:

Table 4 Study drugs

Drug name	Trade name (Manufacturer)	Drug class
Artemether-lumefantrine (AL)	Ajanta Pharma Ltd	Artemisinin derivative and bisquinoline
Primaquine phosphate (PQ)	Government Pharmaceutical Organisation, Thailand	8-aminoquinoline
Placebo	Kampala Pharmaceutical Industries Ltd	Inert, non-active substance

4.6.5.1 ADMINISTRATION OF AL

- Study nurse requests AL from pharmacy

- Study nurse then administers the first artemether-lumefantrine (AL) dose. The dose is dissolved in drinking water in a cup/spoon and administered to the child to drink under observation. The study nurse documents that the dose has been given on participant's medication record and clinic card (Appendix J).

- Study nurse observes the patient for 30 minutes. Any participant who vomits the medication within 30 minutes of administration will be re-treated with a second dose (requested from pharmacy). Any participant who vomits repeatedly (>3 times) will be recorded as complicated malaria and treated according to national guidelines.

- If the participant vomits, the study nurse documents this on the participant's medication record and clinic card.

4.6.5.2 ADMINISTRATION OF PRIMAQUINE/ PLACEBO

- At the same time as the fifth dose of AL, in the morning of Day 2, the study nurse requests the primaquine dose from pharmacy.

- The PQ/ placebo solution (1mg/ml) is prepared by the study pharmacist by dissolving the primaquine tablets according to a standardized SOP. The study pharmacist documents the dose on the treatment allocation form (as above). The pharmacist draws up the dose into a sterile syringe and hands the syringe to the study nurse.

- The study nurse administers the liquid PQ/ placebo to the participant on a spoon. The study nurse documents that the PQ/ placebo has been given on the participant's medication record and clinic card.

- The study nurse observes the participant for 30 minutes. Any participant who vomits the medication within 30 minutes of administration will be re-treated with a second dose (requested from pharmacy). Any participant who vomits the primaquine/ placebo dose repeatedly (>3 times) will be excluded from the study. If there is a possibility that they have ingested any of the primaquine dose, they will be excluded from efficacy analysis, but followed up for safety outcomes and adverse events. If the participant vomits, the study nurse documents this on the participant's medication record and clinic card.

4.6.5.3 ADDITIONAL MEDICATIONS

On the day malaria is diagnosed, patients will receive paracetamol (10mg/kg) to take as needed until the resolution of fever. Patients found to have uncomplicated malaria and a concomitant illness will be treated for both and followed up according to the study protocol. For patients with anaemia (Hb < 10 gm/dL), we will follow Integrated Management of Childhood Illness (IMCI) and Ugandan national guidelines: anaemic children will be treated with iron sulfate (100 mg daily for 2 weeks) and mebendazole (250 mg age 1-2 years; 500 mg > 2 years age; treated no more frequently than every 6 months).

4.6.6 DRUG ACCOUNTABILITY

The medications used in the study will be supplied to the main study office at the IDRC in Mulago Hospital Complex, Kampala. Artemether-lumefantrine will be ordered through the Ajanta Pharma Ltd representative, Surgipharm (Kampala, Uganda). Primaquine is ordered through the Government Pharmaceutical Organisation, Bangkok, Thailand. The medications will be stored as per manufacturers' guidelines. Product inserts and detailed documentation relevant to the procurement of the study medications including batch number and expiry date will be kept in the study regulatory binder.

Study medications will be stored at the study clinic. Monthly inventories of storage conditions and stocks (medications used and remaining) will be kept at the study clinic.

Any unused primaquine after the study will be destroyed according to a protocol agreed with the Government Pharmaceutical Organisation, Thailand.

4.7 FOLLOW-UP EVALUATIONS AND PROCEDURES

Table 5 summarizes the scheduled follow up evaluations and procedures.

4.7.1 LOCATION FOR FOLLOW UP

On each day, participants will attend the clinic in the morning and remain in the study clinic until they have been observed for 30 minutes after their second dose of AL.

On Day 0, they will return home with fieldworkers so that the location of their home can be documented and marked with GPS. This is so that patients can be contacted at home if they do not attend for follow up, in order to reduce loss to follow up (Appendix K).

Unless a patient is unable to attend and the study coordinator considers it appropriate/ possible to follow up at home, all follow up (days 1-28) will be conducted at the study clinic.

4.7.2 HOME VISITS

Individuals who are not well enough or unable to attend the study clinic on scheduled follow up days will be contacted at home and followed up at home if they are unable to come to the study clinic. As far as possible, these participants will be transported to the clinic for clinical care and follow up.

4.7.3 CLINICAL EVALUATIONS

On each day of follow up, a history of presenting complaint will be taken along with a focused physical examination (Appendix L).

4.7.4 BLOOD SAMPLING

On days 0, 1, 2, 3, 7, 10, 14, 21 and 28, a finger prick blood sample for thick and thin smear and filter paper sample will be taken. This will be taken by cleaning a digit with alcohol, then pricking with a lancet and allowing drops of blood to fall onto the following receptacles:

1. Hemocue® cuvette (1 drop)
2. EDTA eppendorf tube (approx 450µl)

If not enough blood is obtainable through finger prick, a second digit will be pricked or a venous sample will be taken.

In the laboratory, the EDTA sample will be mixed, then blood will be extracted using a micropipette to drop onto 2 glass slides for thick and thin malaria films, filter paper (6 drops of 50µl fixed volume), 50µl into L6 buffer (a medium for QT-NASBA samples). On day 0 and 14, an additional 2 drops will be dropped onto filter paper for quantitative G6PD enzyme function assessment (ELISA).

4.7.5 ADVERSE EVENT MONITORING

Assessment for adverse events will be conducted in a systematic fashion at all visits, including the enrollment visit (e.g. vomiting post AL).

At each follow up, study staff will assess participants in an objective manner according to the study clinical record form (Appendix L) so that there is standardization of the assessment and it can be

quantified. Relevant clinical data will be recorded in source documents. If a clinical sign, symptom, laboratory result or event is graded as serious/ severe, then it will be handled as an adverse event (Section 6.7.14).

Table 5 Follow up evaluations and procedures

Day of follow up	0	1	2	3	7	10	14	21	28	Unscheduled
CLINICAL:										
History	X	X	X	X	X	X	X	X	X	X
Tympanic temperature	X	X	X	X	X	X	X	X	X	X
Physical examination	X	X	X	X	X	X	X	X	X	X
Assessment for adverse events	X	X	X	X	X	X	X	X	X	X
Complete case record form	X	X	X	X	X	X	X	X	X	X
TREATMENT:										
ACT	X (1st)	X (2nd)	X (3rd)							
Primaquine (PQ)			X							
LAB TESTING:										
Test	**Sample collected into EDTA tube then pippetted in the lab**									
Blood smear	X	X	X	X	X	X	X	X	X	X
Filter paper W#3 + W#903	X	X	X	X	X	X	X	X	X	X
L6 buffer	X	X	X	X	X	X	X	X	X	
Haemoglobin (Hemocue®)	X	X	X	X	X	X	X	X	X	
G6PD function (Spot test)	X									
G6PD function (ELISA)	X						X			

4.7.6 UNSCHEDULED FOLLOW-UP

Participants will be encouraged to come to the clinic on any day during the study (days 0-28, regardless of whether it is a planned follow-up day) when they require medical attention or they have a question for the study team. The participant will be reimbursed for travel to and from the clinic.

On unscheduled follow up days, when the participant self-presents to the clinic, a history and physical examination will be conducted along with any relevant investigations to determine the cause of presentation. A finger prick blood sample for malaria thick smear and filter paper sample will also be

taken in those with fever. Adverse event monitoring will be conducted and a urine dipstick test will be taken to assess for haemolysis if suspected.

Unscheduled assessments will be documented on a clinical case record form (Appendix M).

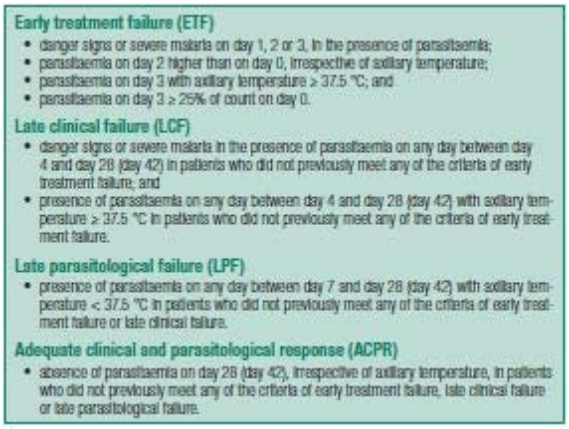
4.7.7 MANAGEMENT OF MALARIA

During follow up (scheduled and unscheduled visits), if there is any evidence of severe malaria, participants will be treated according to national guidelines and IMCI and referred and transferred to Jinja Children's Hospital (the regional paediatric referral hospital) for inpatient care. The Jinja Hospital paediatricians are aware of the study and the transfer time by road is less than 20 minutes. Clinical records, blood slides and other blood results measurements and G6PD results obtained at Walukuba will be provided to the Jinja Hospital clinicians. Study clinicians will accompany the child upon referral and review and complete the case record form of referred patients daily and a hospital follow up record (Appendix N).

4.7.7.1 MALARIA OUTCOME CLASSIFICATION SYSTEM FOR PATIENT MANAGEMENT

For the purposes of clinical management of malaria, treatment outcomes not adjusted for genotyping will be measured using the standard WHO classification system (early treatment failure, late clinical failure, late parasitological failure, and adequate clinical and parasitological response) ("Methods for Surveillance of Anti-malarial Drug Efficacy, WHO 2009, Appendix O).

These will be used to guide clinical case management. These treatment outcomes are not to be confused with the study outcome measurements. They are used purely to guide clinical decisions. The treatments used will be those advised by the Ugandan national malaria guidelines.



Early treatment failure (ETF)

- danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitaemia;
- parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature;
- parasitaemia on day 3 with axillary temperature ≥ 37.5 °C; and
- parasitaemia on day 3 ≥ 25% of count on day 0.

Late clinical failure (LCF)

- danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure; and
- presence of parasitaemia on any day between day 4 and day 28 (day 42) with axillary temperature ≥ 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure.

Late parasitological failure (LPF)

- presence of parasitaemia on any day between day 7 and day 28 (day 42) with axillary temperature < 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response (ACPR)

- absence of parasitaemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

Figure 6 From *Methods for Surveillance of Anti-malarial Drug Efficacy.* WHO, 2009

For the the purpose of case management, clinical decisions will be based on the following scenarios:

1) Danger signs or severe malaria in the presence of parasitaemia on any day of follow up→ treat with parenteral artemisinin/ iv quinine
2) Early treatment failure → treat with quinine or alternative ACT
3) Late clinical failure (day 4-14) → treat with alternative ACT
4) Late clinical failure (day 15-28) →treat as new infection. Treat with AL
5) Late parasitological failure→ treat as new infection. Treat with AL

6) Adequate clinical and parasitological response → no additional treatment

4.7.8 MANAGEMENT OF HAEMOLYSIS

In previous studies where a single dose of 0.75mg/kg primaquine has been administered, the incidence of severe haemolysis has been low. In Tanzania [27], none of the participants experienced symptoms of anaemia and no child required a blood transfusion. In the second study in Tanzania[28], one child who received primaquine 0.75mg/kg had severe anaemia, but did not require a blood transfusion and recovered with haematinic drug treatment.

In Sudan[29], there were no severe or serious or adverse events and severe anaemia was not reported.

Consequently, given that the frequency of G6PD deficiency is likely to be similar in Uganda, and children with low G6PD enzyme function on day 0 are excluded from enrolment we do not expect haemolysis to occur frequently in those participants receiving 0.75mg/kg of primaquine. We predict that those participants in the dose arms lower than 0.75mg/kg should have an even lower chance of developing haemolysis because haemolysis is dose-related.

For the purposes of systematic and responsible safety monitoring, the following detailed protocols have been developed for the management of participants in whom haemolysis is suspected.

4.7.8.1 MEASURES OF HAEMOLYSIS

Haemolysis will be suspected according to criteria in a study SOP, detailing the size of haemoglobin fall (measured by Hemocue®) after PQ/ placebo treatment and the absolute haemoglobin value. In addition, any child presenting with or complaining of dark or black urine will be assessed for haemolysis.

4.7.8.2 INVESTIGATION OF HAEMOLYSIS

If haemolysis is suspected, a venepuncture sample will be taken for a full blood count and G6PD enzyme function, a blood film will be prepared and analysed for schistocytes, urine dipstix will be taken and a clinical examination performed. The procedures for further investigation are summarized in figure 7 below.

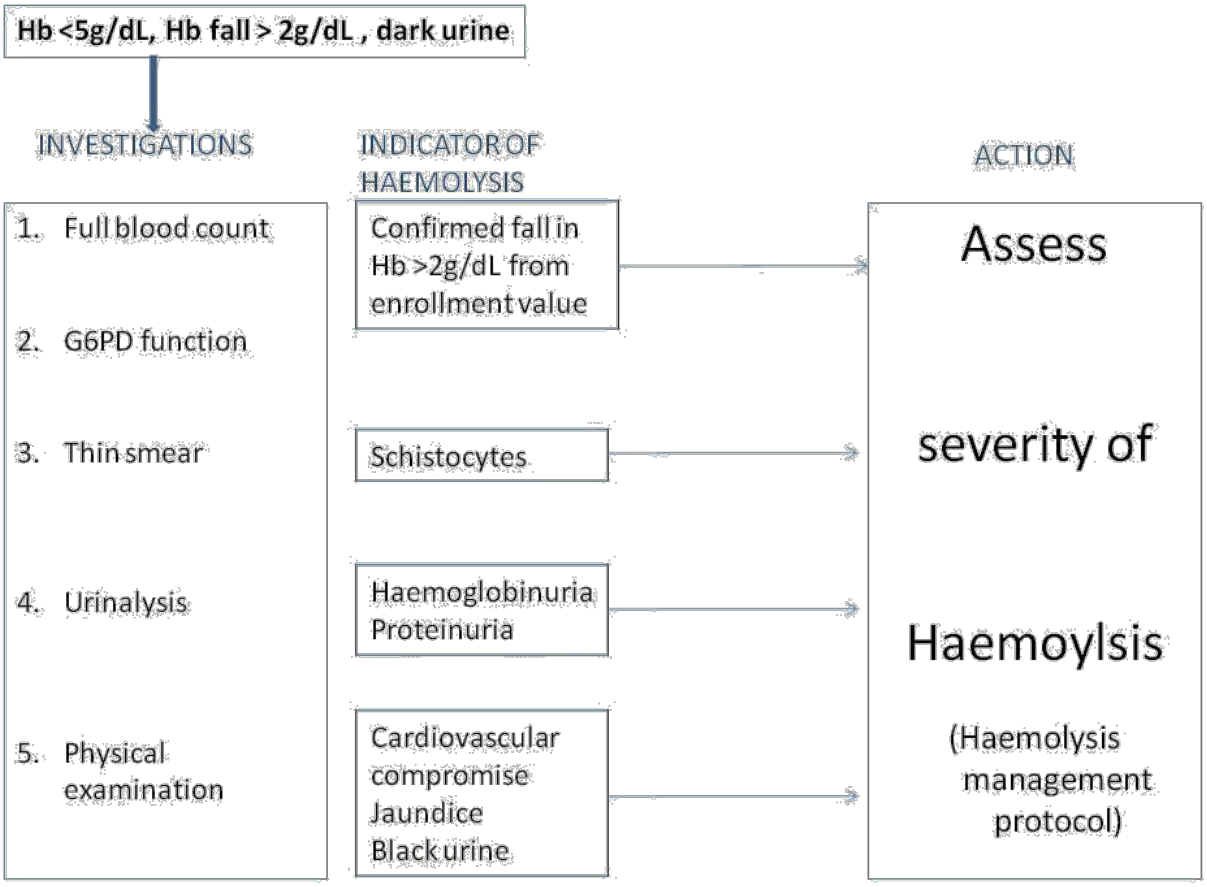


Figure 7 Investigation of suspected haemolysis

4.7.8.3 MANAGEMENT OF ANAEMIA/ HAEMOLYSIS

If a participant shows signs of haemolysis, they will be managed according to the schematic in figure 8.

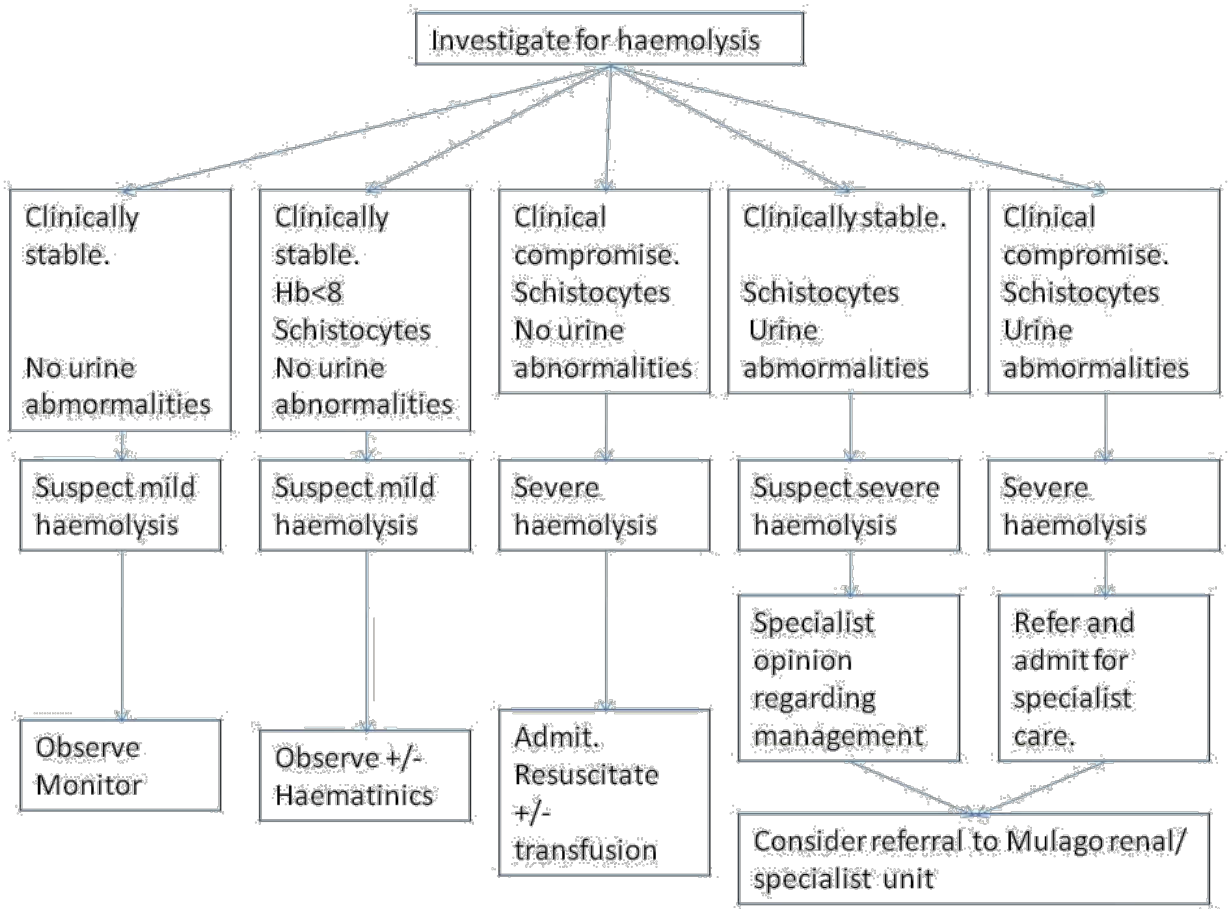


Figure 8 Management of haemolysis

Anaemia

Participants with a haemoglobin below 10g/dL (according to IMCI guidelines) will be treated with haematinic drugs and de-worming according to IMCI and national guidelines.

Haemolysis

Children with evidence of mild haemolysis will be observed and monitored and haematinic drugs will be considered according to IMCI and national guidelines.

Haemoglobin testing will be repeated according to the child's clinical progress.

Any child reporting black urine will be assessed by a study physician. Black urine is defined as any dark-coloured urine with brown or black pigments (not orange). A full clinical exam and history will be obtained, a urine sample sent for analysis (haematuria, protein) and a venous blood sample drawn for assessment of full blood count (FBC) and renal function (including bicarbonate). A blood film will be assessed for schistocytes.

Requirement for blood transfusion will be assessed by physicians, considering the size and rate of Hb drop and signs of clinical compromise, according to IMCI guidelines. If blood transfusion is required, the participant will be transferred to Jinja Children's Hospital.

In addition to routine care in Jinja Children's Hospital, daily follow-up will be provided by study physicians and progress will be documented in the case record form and hospital follow up form (Appendix N).

If a participant is transferred to Mulago National Referral Hospital in Kampala, a study clinician will be in attendance for daily follow-up progress will be documented in the case record form and hospital follow up form (Appendix N).

4.7.9 MANAGEMENT OF NON-MALARIAL ILLNESSES

Participants found to have non-malarial illness in addition to their presentation or during follow-up will be managed at the discretion of the study physician with reference to standard protocols of care. These protocols are used by the IDRC and accepted to be consistent with the standard of care locally. This standard treatment will be given in the study clinic and recorded in the participant's CRF. Where appropriate, referrals will be made to relevant specialist care in Jinja Children's Hospital or Mulago National Referral Hospital.

The routine use of non-study medications with antimalarial activity, including tetracycline, antifolates, and macrolide antibiotics, will be avoided when acceptable alternatives are available. If alternatives are available, new prescriptions of drugs which can exacerbate anaemia, such as trimethoprim, zidovudine and pyrimethamine or drugs which can precipitate haemolysis with G6PD deficiency (Youngster, Drug Safety 2010+++), such as dapsone and nitrofurantoin, will be avoided in all participants. Drugs which might interact with primaquine are penicillamine, and quinacrine. These are not available in this population, but study staff will be made aware to avoid prescribing.

4.7.10 OUT OF HOURS PRESENTATIONS

When participants attend the health facility out of study clinic hours, they will be treated with local standard care. The health facility staff will be requested to refer all out of hours participants to the study clinic the following morning for assessment by the study staff with a written note of out of hours clinical assessment. If they have been admitted, the health facility staff will inform the study staff of the event and study staff will assess and follow up the participant and document the episode as per a non-scheduled visit.

4.7.11 CRITERIA FOR EXCLUSION AFTER ENROLLMENT

For efficacy analysis, participants will be excluded after enrollment for the reasons listed below. All participants who have received the study drug primaquine will be followed up for safety and adverse event outcomes.

1. Repeated vomiting of primaquine on day 2. These patients will be excluded from the efficacy analysis, but they will be followed up for safety outcomes and have adverse event monitoring for the duration of the study (up to day 28), given the chance that they may have absorbed a small amount of study drug.

2. Evidence of serious illness. If, after enrollment and before receiving primaquine/ placebo on day 2, a participant develops signs of severe malaria or another serious intercurrent illness, (e.g. measles, hepatitis) the participant will be excluded and referred for appropriate medical attention. If a participant develops signs of severe malaria or another serious intercurrent illness after receiving the study drug primaquine/ placebo, they will not be excluded from efficacy or safety analysis and will be followed up by the study staff.

3. Withdrawal of consent at any stage. If a participant or their parent/ guardian withdraws consent at any stage of the study, they will be excluded from efficacy analysis and no further follow up will be conducted. They will be discharged to usual health centre care.

4. Loss to follow-up. Participants who do not visit for days 1 and 2 will be contacted at home on the telephone (if available) or followed up by home visit (as far as possible). Considerable effort will be made to ensure the participant does not miss treatment for their malaria infection and attends for the study treatments within 24 hours. If they are not obtainable, this will be termed a missed visit and the participant will be noted as "lost to follow-up" for the purposes of the efficacy analysis. Missed visits after day 2 will be managed according to an SOP with regards safety and efficacy analysis.

4.7.12 PHARMACOKINETIC ANALYSES

4.7.12.1 Overview

Pharmacokinetic evaluations will be obtained on approximately one quarter of the enrolled participants; a maximum of 160 participants will be recruited for pharmacokinetic sampling. There will be a separate consent process for this evaluation. Participants will be consented for this on day 1 and asked to come for sampling on days 2 to 4. The sampling on day 2 will happen whilst they are at the clinic for their last day of AL and the study dose of PQ/ placebo.

The pharmacokinetic sampling will involve taking a total of 7 venous blood samples of less than 2mls. The total amount sampled, being approximately 11-14 mls in 3 days. The first sample is just prior to the PQ/ placebo dose (a baseline sample) and the subsequent six doses are at intervals up to 72 hours after the dose of primaquine/ placebo. The blood samples will be taken at fixed times between 8am to 5pm. Participants will have to attend the clinic a minimum of 30 minutes prior to this to enable preparation for sampling. The first 5 samples are taken on day 2 and they will be taken through a venflon, sited when the baseline pharmacokinetic sample is taken. If a venflon is not sited successfully, a butterfly needle may be used. The last two samples (one on day 3 and one on day 4) will be taken by individual blood draws (venepuncture). The participant will be asked to stay in the clinic between sampling times on day 2.

In order to minimize the total number of blood draws per participant, the sampling timeframe has been randomized so that over the total population of participants, a population pharmacokinetic model can be constructed for analysis. Six randomized sample times will be allocated to sequential consenting participants in opaque envelopes. Each sample time is within a window so that there are 5 samples on day 2 and one each on days 3 and 4.

Pharmacokinetic samples will be analysed in Professor Niklas Lindegardh's laboratory in Mahidol University, Bangkok, Thailand, where the randomized sampling framework was generated.

4.7.12.2 Recruitment and consent to pharmacokinetic sampling

On day 1, every fourth child who was enrolled will be invited to give written informed consent for pharmacokinetic sampling (Appendix P). As far as possible, children will be seen in enrollment order (study number order) on each day. If this child declines consent, the next consecutive participant will be invited. The consent interview will be conducted by study clinicians. The pharmacokinetics consent form will be attached to an information leaflet in the appropriate language and it will be read word for word to the guardian of the child. Children over the age of eight years will be invited to give written assent by signing a form with attached information sheet which is read to them.

4.7.12.3 Selection criteria for pharmacokinetic sampling

All participants undergoing pharmacokinetic sampling must satisfy the following criteria:

Inclusion criteria:

1. Haemoglobin >8g/dl
2. Siting of secure blood sampling access feasible (venflon/ butterfly needle) on day 2
3. Willing and able to attend study clinic by 7.30am on days 2-4
4. Willing to stay on study clinic premises between 8am to 5pm on day 2

4.7.12.4 Pharmacokinetic sampling procedures

Allocating sampling times.

Consenting participants will be seen by a study clinician who will select the next available pharmacokinetic opaque envelope. This will contain a sheet with the sampling times for the participant. The clinician will label this sheet with the participant's study number. The clinician will be responsible for adhering to the sampling times.

Taking blood samples.

The clinician will fix venous access (using a venflon or butterfly) and take the baseline sample. All blood will be taken into heparinised tubes. The subsequent 4 samples will be taken at the allocated sampling times (Table 6). Then the fixed venous access will be removed. The participant will be informed as to what time they need to present by on days 3 and 4 for the last two samples. On these days, the sample will be taken by phlebotomy with no fixed access.

Table 6 Sampling framework for participants recruited to pharmacokinetic studies

Day of follow up	0	1	2	3	4	5	6	7
PQ pharmacokinetic sampling windows:* (baseline plus x 6 windows) Serum samples			0 0-2 2-3 3-6 6-9	24-33	48-72			

*each participant has one sample within each sampling window

4.7.13 LABORATORY EVALUATIONS

4.7.13.1 MICROSCOPY

Microscopy will be conducted by laboratory technicians who are not involved in the clinical care and assessment of study participants. Thick and thin blood films will be stained with 10% Giemsa for 10 minutes. Trained microscopists will calculate asexual parasite density per µl by dividing the number of asexual parasites per 200 white blood cells by 40. If there are less than 10 parasites per 200 WBCs, then they will count the number of asexual parasites per 500 WBC and divide by 16 to calculate a parasite density per µl. Slides will be considered negative if no parasites have been found after counting 500 WBCs. Thin smears will be used for parasite speciation. All routine blood slides will be read within 24 hours. Reading clinically urgent slides will be prioritized over routing slides. Slides will be read by two microscopists for quality control and discrepant results will be confirmed by a third reader.

4.7.13.2 HAEMATOLOGY

During screening, haemoglobin will be assessed with a finger prick blood sample using a HemoCue® photometer (Ängelholm, Sweden). This produces a point-of-care result. If clinically indicated during follow-up, a venepuncture sample will be taken for a full blood count (haemoglobin, white blood cell count, platelets and haematocrit).

4.7.13.3 BIOCHEMISTRY

Where required, for the investigation of adverse events, biochemistry samples may be taken, for example, for renal and liver function testing and urinalysis for haemolysis. These samples will be sent to a commercial laboratory with quality control systems.

4.7.13.4 G6PD DEFICIENCY TESTING

An EDTA tube or heparinized hematocrit capillary tube will be used to acquire blood for glucose-6-phosphate-dehydrogenase semi-quantitative fluorescent spot test for initial screening in the clinic site. This will require approximately 0.5 ml of blood. A reagent solution containing Glucose-6-P + $NADP^+$ is mixed with whole blood or a dried blood spot. Samples obtained from normal or slightly reduced G6PD activity will show strong fluoresce. Failure to fluoresce after 10-minutes of incubation suggests a total or marked deficiency of G6PD. This test may fluoresce falsely if the study participant has had a blood transfusion within the last 90 days hence, these persons will be excluded from the study. In addition,

dried blood spots obtained on filter paper will be stored for quantitative G6PD testing. This will be performed using the G-6-PD OSMMR 2000 kit (R&D Diagnostics). The dried blood spots will also be utilized for G6PD genotyping.

4.7.13.5 MOLECULAR STUDIES

Real-time quantitative nucleic acid sequence-based amplification (QT-NASBA)

Blood will be stored in buffer (L6 buffer) and on filter paper. Nucleic acid will be extracted from blood using the Boom extraction method(1990)[39]. Sub-microscopic gametocyte density will be measured using the *Plasmodium falciparum* 25 S mRNA real time QT-NASBA developed by Schneider [40].

G6PD genotype

Samples will be collected on filter paper for G6PD genotyping using PCR for the common alleles in Uganda and East Africa.

4.7.13.6 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)-PRIMAQUINE PHARMACOKINETICS

Whole blood samples will be collected in heparinised tubes from venepuncture for pharmacokinetic/ pharmacodynamic analysis on those patients who are consented for the procedure. Blood samples will be centrifuged to obtain serum samples.

Samples for pharmacokinetic and pharmcodynamic analysis will be stored at -20 degrees and transported for analysis at Mahidol University, Bangkok, Thailand in the laboratory of Professor Niklas Lindegardh. This laboratory specializes in the pharmacokinetic analysis of antimalarials and HPLC analysis will be conducted for primaquine and relevant metabolites.

4.7.14 ADVERSE EVENT MONITORING

4.7.14.1 DEFINITIONS

4.7.14.1.1 ADVERSE EVENTS

An **adverse event** is defined as any untoward or unfavourable medical occurrence in a human subject, including and sign (such as a laboratory finding), symptom or disease and including errors in clinical management of the participant (e.g. dosing errors) which is temporally associated with the subject's participation in the research, whether or not considered related to the subject's participation in the research (modified from the definition of adverse events in the 1996 International Conference on Harmonization E-6 Guidelines for Good Clinical Practice).

4.7.14.1.2 SERIOUS ADVERSE EVENTS

A **serious adverse event** is any untoward medical occurrence which poses a threat to the participant's life or functioning as follows:

-Results in death

-Is life-threatening (i.e. the participant was at risk of death at the time of the event)

-Requires inpatient hospitalization or prolonged hospitalization beyond the expected stay

-Results in persistent or significant disability/ incapacity -Is a congenital anomaly/ birth defect

-Requires a medical or surgical intervention to prevent one of the outcomes listed above

4.7.14.1.3 UNEXPECTED ADVERSE EVENTS

An **unexpected adverse event** is one which has not previously been observed, i.e., is not in the available product information irrespective of whether or not it is theoretically possible given the pharmacological properties of the study medication.

A **Suspected Unexpected Serious Adverse Reaction (SUSAR)** is one which interpreted as a response to the medicinal product and is of severity or a nature which is not consistent with the product information.

4.7.14.2 IDENTIFICATION AND RECORDING OF ADVERSE EVENTS

Participants will be monitored for adverse events on each day of scheduled follow up and on unscheduled follow up visits. This will involve the identification of any new signs or symptoms that were not present on the previous visit.

Adverse events will be recorded on a separate adverse event reporting form (Appendix Q). The following data will be collected on adverse events:

-Description of adverse event
-Date of adverse event onset
-Date adverse event reported
-Maximum severity of the adverse event
-Maximum suspected relationship of the adverse event to the study medication
-Is the adverse event serious?
-Is the adverse event unexpected?
-Identification of the person reporting the adverse event
-Was the event episodic or intermittent in character? -
Outcome of the adverse event
-Date of resolution of the adverse event

Duration of follow up: Adverse events will be followed up until they have resolved or stabilized in the opinion of the study clinician, even in the event that this exceeds the end of the study or following a patient's withdrawal from the study.

4.7.14.3 GRADING OF SEVERITY OF ADVERSE EVENTS

The severity of adverse events (symptoms, signs, abnormal laboratory parameters) will be graded according to a system developed by the UMSP/ IDRC[41] which are in accordance with guidance from the NIH Division of Microbiology and Infectious Diseases (DMID) toxicity tables (http://www.niaid.nih.gov/LabsAndResources/resources/DMIDClinRsrch/pages/toxtables.aspx) and the WHO Toxicity grading scale for determining the severity of adverse events. The grading of severity of adverse events is summarized in Appendix R. The causal association of adverse events with use of study medication is summarized in Appendix S.

4.7.14.4 REPORTING OF SERIOUS ADVERSE EVENTS

Periodic summaries of all adverse events will be compiled by the principle investigator and submitted to the DSMB. Reporting of serious adverse events, fatal/ life-threatening events will be according to the requirements of the IRBs (SOMREC, LSHTM, UNCST) and the NDA.

5.0 STATISTICAL ISSUES

5.1 SAMPLE SIZE

The number of participants required in each treatment arm was calculated for each of the two primary outcome measures:

1. EFFICACY PRIMARY OUTCOME MEASURE: number of days to gametocyte clearance (gametocyte clearance time, GCT)
2. SAFETY PRIMARY OUTCOME MEASURE: maximal fall (+/ or -) in haemoglobin (g/dL) from enrollment to day 28 of follow-up

Sample size for efficacy

For efficacy, the sample size calculation is based on non-inferiority of each of the two test dose arms to the comparator arm, the WHO-recommended dose of PQ, 0.75mg/kg.

The non-inferiority margin for days to gametocyte clearance is proposed as 2.5 days, taking into consideration data from previous studies. The addition of primaquine to ACT in Tanzania reduced the time to gametocyte clearance from 28.6 to 6.3 days. We used the size of this difference to consider a clinically acceptable inferiority margin.

The standard deviation for time to gametocyte clearance is estimated as 6 days, using data from Bousema 2010 [26] with adjustment for the fact that re-infection was not accounted for in the 28 day follow up period.

Applying these assumptions, and allowing for a 10% loss to follow up, a sample size of 120 per arm will provide over 80% power at the 0.05 significance level to detect non-inferiority to the standard arm with a non-inferiority margin of 2.5.

This sample size also allows for an analysis of superiority of the efficacy of the two test dose arms to placebo.

Sample size for safety

For safety, the sample size calculation is based on superiority of each of the two test dose arms to the comparator arm, the WHO-recommended dose of PQ, 0.75mg/kg.

From Bousema 2010[26], the overall mean absolute drop in Hb by day 7 after treatment with ACT/PQ was 0.6g/dL with a standard deviation of 1.5. Therefore, with 80% power and at the 0.05 significance level, a sample size of 99 would be required to detect a difference in mean maximal drop in Hb between treatment groups of 0.6g/dL.

Therefore, a total study size of 480 will be required to analyse both of the primary outcomes.

5.2 ANALYTICAL PLAN

For each treatment group, the numbers of participants who were randomised, received each dose of the intended treatment, and were analysed for the primary outcome will be represented in a CONSORT flow chart[42].

Baseline characteristics of each arm will be tabulated.

5.2.1 Analysis for primary efficacy outcome:

The mean and standard deviation of the number of days to gametocyte clearance (gametocyte clearance time; GCT) will be estimated in each treatment arm by use of a mathematical model[43].

Non-inferiority of test treatments compared to standard treatment

For each of the two test PQ dose treatment arms, PQ1 and PQ2, a 95% confidence interval for the difference in mean GCT between the test arm and the WHO-recommended PQ dose, PQ-R, treatment arm will be calculated (mean GCT in test arm – mean GCT in PQ-R arm). Figure 9 demonstrates the possible scenarios of treatment differences in relation to the non-inferiority margin of 2.5 days, and how each would be interpreted. For example, if the upper limit of the 95% confidence interval is less than 2.5 days, then the conclusion would be that the test treatment is non-inferior to the comparison treatment. If the upper limit of the 95% confidence interval is greater than 2.5 days but the lower limit is less than

2.5 days, then no conclusion on non-inferiority of the test treatment to the comparison treatment could be made. If both the lower and upper limits of the 95% confidence interval are greater than 2.5 days the conclusion will be that the test treatment is inferior to the comparison treatment.

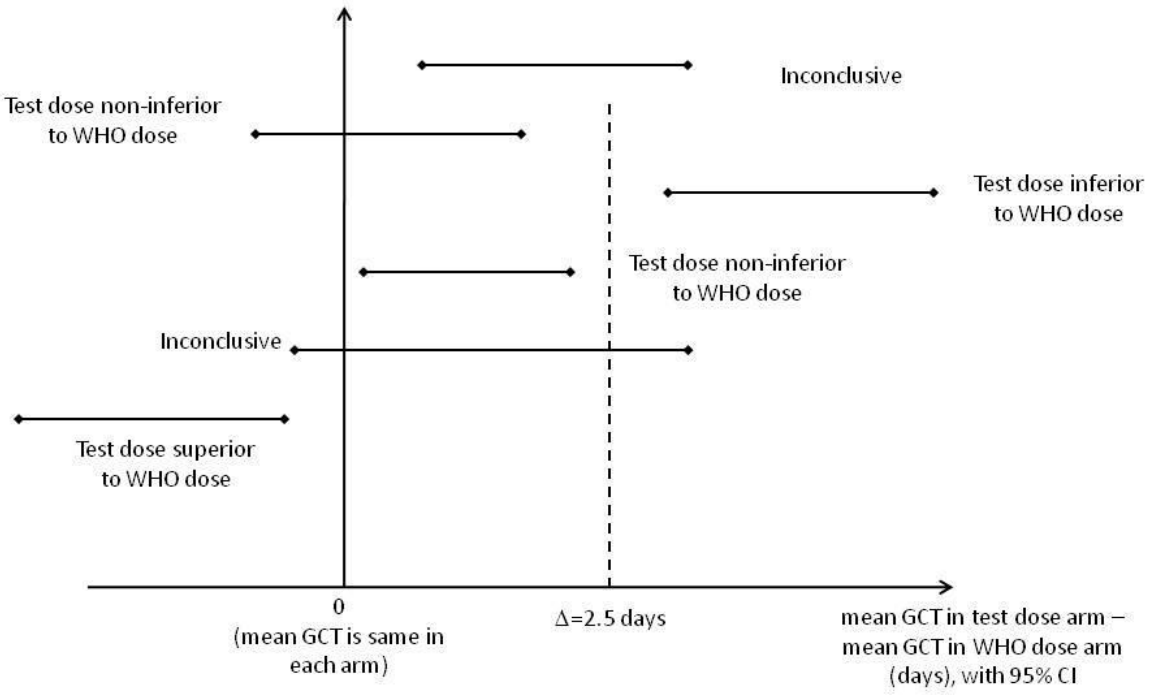


Figure 9 Possible scenarios and interpretation of non-inferiority analysis

Superiority of test treatments compared to placebo

For each of the two test arms, superiority over the placebo arm will be assessed using unpaired t-tests. Differences between means and 95% confidence intervals for the differences will be calculated.

5.2.2 Analysis for primary safety outcome:

The primary safety outcome, maximal fall (+/ or -) in haemoglobin (g/dL) compared to enrolment value during follow-up, is expressed as an arithmetic mean (+/- standard deviation) per treatment arm and pair-wise comparisons made between each of the two test treatment arms, PQ1 and PQ2, and the comparator (WHO-recommended) arm, PQ-R, using unpaired t-tests. Differences between means and 95% confidence intervals will be calculated.

5.2.3 Analysis for secondary efficacy outcome:

For each participant, the mean AUC of sub-microscopic gametocyte density over time per day (meanAUC/day) will be calculated using the linear trapezoid method: meanAUC/day from days 0 to 28 is calculated as

$$meanAUC/day = [(2-0)\times(g0+g2)/2 + (3-2)\times(g2+g3)/2 + (7-3)\times(g3+g7)/2 + (10-7)\times(g7+g10)/2 + (14-10)\times(g10+g14)/2 + (21-14)\times(g14+g21)/2 + (28-21)\times(g21+g28)/2]/28$$

where *gd* represents gametocyte density on day d.

The distribution of mean AUC/day is likely to be skewed, therefore the geometric mean (+/- standard deviation) of the mean AUC/day will be calculated for each treatment arm.

(1) Non-inferiority of test treatment arms to reference arm

The reference treatment arm, PQ-R, will be the WHO-recommended dose of PQ, 0.75mg/kg (plus ACT). The proposed non-inferiority margin for the difference between the means of test doses and mean of reference arm is 0.2. Confidence intervals for the difference between the means will be calculated.

(2) Superiority of test treatment arms to placebo

Two pair-wise comparisons of the test doses arms, PQ1 and PQ2, with the placebo arm will be made using the unpaired Student's t-test with p values of 0.05 taken as significant.

The anti-logAUC value will be used to give the pair-wise comparisons between the treatment arms as geometric mean ratios with 95% confidence intervals.

5.2.4 Subgroup analyses

Analysis stratified by gametocyte prevalence and by density at enrolment will also be presented.

The primary and secondary efficacy and safety outcomes will be compared in the following groups (listed below) for each treatment arm and overall. Where outcomes are binary, they will be analysed using logistic regression, while continuous outcomes will be analysed using linear regression. Interaction terms will be fitted in regression models to assess whether the effect of each treatment compared is modified by each of the factors listed below.

-Male vs female

-Age<5 vs Age ≥ 5 years

-Baseline Hb< 10 g/dL vs Baseline Hb≥10g/dL

5.2.5 Pharmacokinetic analysis

Population pharmacokinetic modelling analysis is under design by collaborators in Mahidol Oxford Research Unit, Bangkok, Thailand. The sampling framework has been optimised to minimise sampling points, to prevent the need for overnight stay at the clinic and to produce basic pharmacokinetic parameters including: AUC of concentration over time, T_{max}, C_{max}, oral clearance, terminal half life of primaquine +/- metabolites.

5.2.6 Additional analyses and management of missing data

The above analyses will be undertaken as "intention-to-treat" (including all individuals randomized). Since ITT analysis may increase the risk of falsely claiming non-inferiority, a "per-protocol" analysis (including all individuals followed up as per protocol) will also be undertaken.

Missing data will be accounted for in the analysis. Weight will be placed on specific data points such that if crucial data points are missing, this will exclude a participant from per-protocol analysis. Sensitivity of primary outcomes to missing data will be assessed.

Timing for any interim analysis will be fixed by the DSMB prior to starting the study.

6.0 MONITORING

6.1 DATA AND SAFETY MONITORING BOARD

A data and safety monitoring board, will review the study protocol prior to implementation of the trial and will be convened to review the study periodically. The agenda for each meeting will be made in conjunction with the Clinical Trials Unit (CTU) at LSHTM and the DSMB Chair. The CTU is responsible for quality assurance in clinical trials sponsored by LSHTM.

6.2 MONITORING PLAN

All study data and interim results will be presented to the DSMB using treatment group codes (A, B, C, or D) that will correspond with, but not identify, the actual treatment groups. Master copies of the randomization code and treatment group assignments will be held in the administrative offices in Kampala and London.

The timing of interim analysis is expected to be after 250 patients are enrolled and will be confirmed with the DSMB.

Information reflecting study progress and data quality and safety and tolerability data will be provided to the DSMB at regular intervals determined by the DSMB.

An external monitoring visit will be arranged with the LSHTM Clinical Trials Unit (the Sponsor) clinical trials Quality Manager. Monitoring visits at Walukuba Health Centre IV will be conducted alongside a comparison between a selection of CRFs and the study database to ensure accuracy of the data.

6.3 STOPPING GUIDELINES

Guidelines for stopping the study due to safety outcomes will be developed and established by the DSMB.

Interim/cumulative safety data will be made available to the DSMB for review in accordance with the schedule they recommend.

7.0 DATA COLLECTION AND MANAGEMENT

7.1 DATA MANAGEMENT

All clinical data will be recorded onto standardized case record forms (Appendix L, M, N) by study physicians. Laboratory data will be recorded in a laboratory record book by the study laboratory technicians. Data will be transferred from the case record forms and laboratory records into a computerized database and will be double-entered to verify accuracy of entry. Adverse event data will be transferred onto standardized data extraction forms (Appendix Q) prior to entry into the database. Back-up files of the database will be stored as zip files on external hard drive or compact discs after each data entry session. After each week, new data will be sent to the server at UMSP, IDRC for secure storage and back up. For quality control, query programs will be written into the database to limit the entry of incorrect data and ensure entry of data into required fields.

7.2 DATA QUALITY ASSURANCE AND MONITORING

All members of the study team will be educated in the study protocol prior to the onset of the trial and training/ education sessions will continue for the duration of the trial. Knowledge of the study protocol and procedures will be assessed and documented with a post-training questionnaire. The study physicians and nurses will complete case record forms at each patient visit. These forms will be reviewed by the study coordinator for completeness and accuracy. To optimize the quality of thick blood smear slide readings, each slide will be read by two experienced microscopists who will be blinded to the patient's treatment group. Any discrepancies in slide readings will be reviewed and resolved by a third microscopist. Molecular studies and G6PD ELISAs will be conducted in duplicate with positive and negative controls. Study group meetings will be conducted regularly to review the progress of the study, address any difficulties, and provide performance feedback to the members of the study group.

7.3 RECORDS

Individual case record forms will be provided for each subject (Appendix L, M, N). Participants will be identified by their study identification number on study documents and patient names will not be entered into the computerized database. All participant record forms will be kept in individual files in a secured filing cabinet in the study clinic. All corrections will be made on case record forms following GCP guidelines by striking through the incorrect entry with a single line and entering the correct information adjacent to it. All corrections will be initialed and dated by the investigator making the correction. Additional clinical records will be kept in the participant's file. The investigators will cooperate with all requested monitoring visits, audits, or IRB or DSMB reviews.

8.0 PROTECTION OF HUMAN PARTICIPANTS

8.1 ETHICAL CONSIDERATIONS

All participants will receive optimal management of their asexual parasitaemia, with artemisinin combination treatment, artemether-lumefantrine, as per the Ugandan National malaria treatment guidelines.

This study is designed to assess efficacy and safety of different doses of primaquine treatment for clearance of gametocytes, the sexual parasitaemia. Additional treatment of the sexual parasitaemia with primaquine or placebo has no additional benefit for the individual at the point of treatment because gametocytes are harmless to the individual. Primaquine is given in falciparum malaria infections because, by clearing gametocytes, it has the potential to reduce malaria transmission at the community level. This trial is not designed to assess reduction of transmission at the community level. Studies with such aims may follow on from this trial. This is explained to participants in the informed consent process.

Primaquine has no effect on the asexual parasitaemia in *P. falciparum* infection (it is the asexual parasitaemia which causes morbidity and mortality). Individuals receiving placebo (0mg/kg of primaquine) will still have optimal treatment of their clinical malaria infection with AL.

8.2 INSTITUTIONAL REVIEW BOARD

This protocol and the informed consent documents will be reviewed and approved by all institutional review boards (IRBs) before the study begins. Any amendments or modifications to this material will also be reviewed and approved by the IRBs prior to implementations. The IRBs will include Makerere University School of Medicine Research and Ethics Committee (SOMREC), Uganda National Council of Science and Technology (UNCST) and the London School of Hygiene & Tropical Medicine (LSHTM) Ethics Committee.

8.3 RISKS AND DISCOMFORTS

8.3.1 PRIVACY

Care will be taken to protect the privacy of subjects and parents/guardians, as described in this protocol. However, there is a risk that others may inadvertently see patients' medical information, and thus their privacy compromised.

8.3.2 FINGER PRICKS AND VENEPUNCTURE

Risks of these procedures include pain, transient bleeding and soft-tissue infection.

8.3.3 RISK OF STUDY MEDICATIONS

8.3.3.1 RISK OF ARTEMETHER-LUMEFANTRINE

AL (Coartem; Novartis) has been extensively studied through Good Clinical Practice (GCP) standardized preclinical and clinical trials and was added to the WHO Essential Medicines List in 2002 (WHO Roll Back Malaria Treatment Policy). Artefan (Ajanta Pharma Ltd) is on the WHO list of Prequalified Medicinal Products. As with other artemisinins, artemether is characterized by rapid antimalarial action, however, recrudescence is frequent when artemether is provided as a single agent, unless given for at least 5-7 days [44-45]. Lumefantrine also has a high

cure rate, but parasite and fever clearance is slower than with artemether [46]. Artemether-lumefantrine benefits from co-formulation, to improve compliance, and the three-day course is a highly effective antimalarial regime.

The drug appears to be very well tolerated, especially in comparison with other antimalarials and antimalarial combinations. A clinical safety review of children under 12 years of age showed that the most common adverse events were abdominal pain, cough, anorexia, headache, vomiting, and diarrhea (all seen in 5-12% of subjects, Novartis, Coartem monograph, 3rd ed. 2004).

An integrated review of toxicity [47] in 1869 patients (611 under age 13) showed the most commonly reported adverse events were gastrointestinal disturbances (abdominal pain, anorexia, nausea, vomiting diarrhea), headache, and dizziness. Rash and pruritis were reported in <2% of patients. No serious or persistent neurological toxicities were linked to AL therapy. Of 20 severe adverse events in 1869 patients, 19 were likely attributable to underlying malaria or concomitant illness, and one was possibly related to AL use (hemolytic anemia in a 35-year-old 13 days after the last administered dose). One concern addressed in studies of AL was possible cardiac arrhythmogenic potential, based on similarities in the chemical structures of lumefantrine and halofantrine. Halofantrine can cause defects in cardiac conduction, particularly a marked QT prolongation that can produce arrhythmias. In 713 patients treated with lumefantrine and followed with serial electrocardiograms, no adverse clinical cardiac events were recorded. Although trials have been limited to date, no serious cardiotoxicity or neurotoxicity has been reported with the use of AL [46, 48].

8.3.3.2 RISK OF PRIMAQUINE

Methaemoglobin is the product of the oxidation of the haemoglobin iron core, oxyhaemoglobin. This molecule is usually stable and auto-oxidises at a rate of 3% daily. In the presence of oxidative stress, the rate increases and methaemoglobin accumulates. Primaquine induces the formation of methaemoglobin at a higher rate than usual[11]. When the percentage of methaemoglobin exceeds 10% of the normal haemoglobin level, cyanosis can occur. Cyanosis with primaquine is transient and dose-related[49]. There is a lack of evidence on the clinical significance of methaemoglobinaemia with a single dose of primaquine.

In individuals with G6PD deficiency, primaquine causes transient, dose-dependent haemolysis[50]. It is likely that this is due to the effect of one of primaquine's metabolites and that it is mediated through oxidative stress, but the exact mechanism is as yet unknown. In a mass screen and treatment programme in Tanzania[28], in asymptomatic parasitized children aged 1 to 12 years, the mean change in haemoglobin after a single dose of 0.75mg/kg primaquine in combination with sulphadoxine-pyrimethamine artesunate treatment was -0.58g/dL. In G6PD heterozygotes, the mean change in haemoglobin was -1.6g/dL and in homozygote/ hemizygote deficient children, the mean change in haemoglobin was -2.5g/dL. One child had severe anaemia by haemoglobin measurement (4.8g/dL) but their G6PD status was not reported. No child required a blood transfusion.

In a Tanzanian study where 0.75mg/kg primaquine was given to children aged 3 to 15 years with clinical malaria, the mean fall in haemoglobin was 5.2% from enrolment value and this was found on day 7 after primaquine was administered[27]. No child required a blood transfusion and no child had symptomatic anaemia.

In all individuals, primaquine causes abdominal symptoms in a dose-dependent manner. These include abdominal pain, nausea, vomiting and mild diarrhea. These side effects are avoided if primaquine is taken with food[19].

Following a course of primaquine for vivax malaria (30mg for 14 days), there is a single case report of depression and psychosis[51] and a single case report of confusion and hallucinations [52] in the literature.

8.4 TREATMENT AND COMPENSATION FOR INJURY

The usual services offered at the study clinic and at Walukuba Health Centre and Jinja Children's Hospital will be available in case of any injury related to the study. Care will be provided free of charge for injuries or adverse drug reactions related to study participation using available funds.

8.5 ALTERNATIVES

Individuals whose parents or guardians *choose* not to participate in this study will not be enrolled. They will receive standard care for medical problems as they arise at the study clinic or other medical facilities in Jinja.

8.6 COSTS TO THE SUBJECT

There will be no cost to the patients or their parents/guardians for participation in this study.

8.7 REIMBURSEMENT OF COSTS TO THE SUBJECT

Subjects will not be paid for their participation in the study. We will provide all routine medical care, including evaluations and medications available in our clinic free of charge, and we will reimburse participants for the costs of all transportation to and from the study clinic (one round trip repaid at $2.60, which is 7000 Ugandan shillings at the time of writing). In addition, we will reimburse the cost of consultation for referrals made by study physicians to other clinics and services. We anticipate reimbursing the cost of most diagnostic tests (including laboratory test, X-rays, and ultrasounds) and medications resulting from these referrals, using available funds. However, reimbursement of all diagnostic tests and treatment recommended outside the study clinic cannot be guaranteed in all circumstances.

When individuals and their parents/ guardians have to attend the clinic for follow-up visits, they will be compensated for the cost of travel to and from the study clinic using estimates provided by home visitors and information gathered during a household survey of the study area which was conducted by the Uganda malaria Surveillance Project in 2010.

8.8 CONFIDENTIALITY OF RECORDS

Parents and guardians will be informed that participation in a research study may involve a loss of privacy. Care will be taken to protect the privacy of subjects and parents/guardians.

All records will be kept as confidential as possible with hard copies in locked filing cabinets and electronic data on password-protected computers and back-up drives. Participants will be identified primarily by their study number and their names will not be entered into the computerized database. No individual identities will be used in any reports or publications resulting from the study.

Data will be stored for the duration required by the IRBs. The principal investigator will be responsible for the security of records and project documents. For the purposes of GCP, trial regulators may be granted access to study documents.

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10.0 APPENDICES

APPENDIX A WHO SEVERE MALARIA CRITERIA AND DANGER SIGNS

WHO CRITERIA FOR SEVERE MALARIA 2010 (From "Guidelines for the management of malaria")

Clinical features:

- impaired consciousness or unrousable coma
- prostration, i.e. generalized weakness so that the patient is unable walk or sit up without assistance
- failure to feed
- multiple convulsions – more than two episodes in 24 h
- deep breathing, respiratory distress (acidotic breathing)
- circulatory collapse or shock, systolic blood pressure < 70 mm Hg in adults and < 50 mm Hg in children
- clinical jaundice plus evidence of other vital organ dysfunction
- haemoglobinuria
- abnormal spontaneous bleeding
- pulmonary oedema (radiological)

- hypoglycaemia (blood glucose < 2.2 mmol/l or < 40 mg/dl)
- metabolic acidosis (plasma bicarbonate < 15 mmol/l)
- severe normocytic anaemia (Hb < 5 g/dl, packed cell volume < 15%)
- haemoglobinuria
- hyperparasitaemia (> 2%/100 000/µl in low intensity transmission areas or > 5% or 250 000/µl in areas of high stable malaria transmission intensity)
- hyperlactataemia (lactate > 5 mmol/l)
- renal impairment (serum creatinine > 265 µmol/l).

Danger signs

- Less than 3 convulsions over 24 hour period
- Inability to sit up or stand
- Vomiting everything
- Unable to breastfeed or drink
- Lethargy

APPENDIX B SCREENING AND ENROLMENT PROCESS

APPENDIX C INFORMED CONSENT FOR PARTICIPATION IN STUDY

Appendix C
Informed consent form for participation in research

Protocol title:	Evaluation of the efficacy and safety of Primaquine for clearance of gametocytes in uncomplicated falciparum malaria
Source of funding:	The Wellcome Trust
Sponsor:	London School of Hygiene and Tropical Medicine
Site of Research:	Walukuba Health Centre IV, Jinja, Uganda
Principle investigator:	Dr Alice C. Eziefula, MBBS MA MRCP MRCPath
Date:	10th May 2011

PURPOSE OF THE STUDY

This information is being read to you in order to ask you whether you will let your child/ the child under your care participate in this research study. We will now give you information about the research study so that you know what will be involved. We will explain the purpose of this study, how the study will be done, and any risks and benefits. You can ask questions at any time. After this consent form is read to you, and your questions have been answered, you will decide if your child or the child under your care will participate in the study. Medical research includes only people who choose to take part. So it is your choice whether your child or the child under your care will take part. Take your time to make your decision about participating. If you agree for your child or the child under your care to participate in this study, we will ask you to sign this consent form. You will get a copy of this form to keep. If at any time you change your mind about your child/ the child under your care participating in the study, then your decision will be respected.

This study is being done by researchers from universities in Uganda (Makerere University and the Uganda Malaria Surveillance Project) and the United Kingdom (London School of Hygiene and Tropical Medicine) and Thailand (Mahidol Oxford Research Unit). This study is being done to find how well a drug called primaquine works and how this drug can affect a child's health. The WHO (World Health Organisation) says that primaquine should be used to stop malaria spreading from people to mosquitoes. People get malaria when they are bitten by a mosquito infected with malaria. Mosquitoes get infected with malaria when they bite a person who has malaria. Malaria goes from mosquitoes to

people to mosquitoes to people. Primaquine helps stop malaria by preventing people who have malaria from passing malaria on to a mosquito. It does not affect the malaria illness in the individual person who already has malaria, but primaquine stops malaria getting into mosquitoes in that person's home and community, so it stops other people getting malaria from mosquitoes. Primaquine has not been used before to stop malaria spreading in Uganda, but it has been used in other countries in East Africa and many other countries in the world. It has also been used in Uganda for other purposes.

Primaquine is not a new drug, it has been used for many years. The WHO recommends how much primaquine should be given to block malaria from getting from people to mosquitoes. High doses of primaquine, give more chance there of it causing side effects. The main side effect of primaquine is having a lower amount of blood in the body. We are interested to know if lower doses of this drug can also stop malaria getting from people to mosquitoes so we are conducting this study to look at how well lower doses work. We want to know how big the chance of side effects is with the normal dose and with lower doses of primaquine in children in Uganda.

HOW THE STUDY IS DONE

Any child over the age of one year and under ten years who comes to the health centre with a fever and whose blood slide is positive for malaria parasites is invited to take part and be enrolled in the study, as long as there are no signs of serious illness. Approximately 500 children will be enrolled in the study. All children will be treated for malaria with artemether-lumefantrine. This is the malaria treatment advised by the Ugandan Ministry of Health. The study staff will observe all doses of malaria treatment to ensure that it has all been taken. On the last day of malaria treatment, children will be given a single dose (it is given only once) of the study medication, primaquine, which stops malaria getting from people to mosquitoes. Four different doses are being tested: the usual dose, ~~and~~ or two lower doses ~~and~~ or one dose which contains no primaquine at all. Children will be given one of the four doses. Since there are four different doses, this means that the participating child has a one in four chance of being given any one of those doses. Which one of these doses your child gets will be left to chance in a process like a lottery.

After your child has had the study treatment, you will be asked to come back to the clinic for more tests to see how well the drug worked to stop the chance of mosquitoes getting malaria and what effects it has had on your child's health, in terms of safety.

Pregnant females cannot participate in the study. Your child may have a pregnancy test if the study clinicians consider it is necessary to be sure your child is not pregnant.

DURATION OF PARTICIPATION IN THE STUDY

Your child will be enrolled on the day that malaria is diagnosed at the health centre. After that, they will be asked to attend the clinic for eight more visits in the first month after the malaria infection was diagnosed.

PROCEDURES

Enrollment

On the day that malaria is diagnosed, your child will be seen and examined by a doctor and a blood sample will be taken from your child's finger or arm. The blood is tested to find the amount and type of malaria parasites in the blood and the amount of blood (haemoglobin). This is done to help answer the research questions. We will also test the blood for "G6PD deficiency", which can make the blood count fall if primaquine is given. If we find your child has this, they will not be enrolled in the study.

Immediately after the blood samples are taken, your child will be given the standard best treatment for malaria (artemether-lumefantrine). You will be asked to stay in the clinic until your child has had his/ her second dose, then a field worker will accompany you home so that the study staff can make a note of where you live. You are asked to come back to the clinic so your child can be observed whilst taking their malaria treatment on the next two days.

On the third day of malaria treatment, your child will be given the study treatment (primaquine) together with the last doses of the malaria treatment. Your child will be checked by the study doctor and another blood sample will be taken for malaria parasite testing and and the amount of blood in the body.

On all the other days, your child will be checked by the study doctor and also have a blood sample taken, to measure the malaria parasites in the blood and the amount of blood in the body.

Follow up visits

Your child will be asked to attend the clinic for eight more visits after today. Also, you are free to attend the clinic on any extra day if your child has any medical problems or if you have questions during the time of the study. We will ask you for a contact telephone number if you have one so that we can contact you if you forget to come for follow up visits. The study clinic is open between 8am and 5pm and if problems occur out of these hours, you should attend the regular emergency services at Jinja Regional Childrens' Hospital. If you visit outside of the hours of 8am and 5pm, please inform the Jinja Regional Children's Hospital staff that your child is in this primaquine study so that study staff can attend to your child as soon as possible. You can show them your study clinic card.

The follow up visits at the study clinic are on these days after malaria has been diagnosed:

Day 1, 2, 3, 7, 10, 14 (2 weeks), 21 (3 weeks), 28 (4 weeks).

On each follow up visit, your child will be checked by the study doctor and also have a blood sample taken, to measure the malaria parasites in the blood and the amount of blood in the body. Additional blood tests will only be taken if the study doctor feels it is appropriate for the clinical care of the child.

If the study clinician finds any sign of severe illness or new illnesses, your child will be treated according to best local standards of care. Being in the study will not hamper the treatment of medical conditions.

RISKS AND DISCOMFORTS

Blood tests: Your child will have a blood test on each visit to the study clinic (9 times in one month). On the first, the blood will be drawn from a vein. On other days, it will be drawn from a finger or heel prick. If the finger prick does not yield enough blood, then blood will be taken from a vein. The risks of drawing blood include temporary pain from the needle point, bruising, and skin infection. The amount of blood removed will be too small to affect your child's health.

Randomization: There are four different doses of the study medication (primaquine) and your child will be given one of these doses by random chance. The dose that your child receives does not affect your child's recovery from malaria (that is treated with AL), only the chance that your child could pass malaria on to a mosquito, or give more or less side effects than other doses, but this will not be known to you or the study staff treating your child until after the study is completed.

Study medication: The study medication (primaquine) can have the following effects on the body: gastrointestinal effects (e.g. abdominal pain, nausea, vomiting, diarrhoea), headache, dizziness and in individuals with G6PD deficiency it can cause anaemia (low amount of blood in the body) or tea-coloured urine. We will monitor carefully for any signs of these problems and treat if necessary. The chance of your child getting serious blood cell damage is less than 1 in 100.

Unknown risks: Primaquine treatment has been used for many years, but it may have side effects that no one knows about yet. The researchers will let you know if they learn anything that might make you change your mind about your child's participation in the study.

Confidentiality: Taking part in the study may involve a loss of privacy, because we will collect information about your child's health in a record on paper and on a computer, but information about your child will be handled as confidentially as possible Only the people working on the study and researchers with permission will see it. During the study period, these records will be kept at the Walukuba study office and the IDRC offices at Mulago Hospital. Dr. Eziefula will be responsible for the use, storage, and disposal of records. Records will be kept as private as possible. People responsible for making sure that the research is done properly may ask to see your child's records. If you sign this consent form, you are allowing your child's records to be seen by these people.

BENEFITS

All children taking part in the study will have their malaria infection ~~is~~ treated with the best treatment available, in accordance with the Ugandan national guidelines. There is no personal immediate benefit to your child from the study medication (primaquine). The medication is given to reduce the chance that that your child can pass malaria on to a mosquito, reducing the risk of that mosquito passing on malaria to other people at home or in the local area.

The results of the study may be used to decide if in the future, primaquine might be used to reduce malaria in the community.

COST/ PAYMENT

You will not be charged for any of the study treatments or procedures. You will be responsible for all the normal costs for your child's routine health care. You and your child will not be paid for participation in the study.

The cost of transport of your child to and from the study clinic for follow up visits or for any extra visits during the time your child is in the study will be reimbursed to you. The sum of 7000/- UGX (equivalent to $2.60) will be given at each clinic visit to cover the journey home and the next return visit from home to the clinic.

ALTERNATIVES TO PARTICIPATION

Participation in this study is completely voluntary. If you decide you do not want your child to participate in the study, this will not affect your child's care at local clinics or at Jinja Children's Hospital or Walukuba Health Centre. During the study, you will be informed promptly of any new information that may influence your willingness to stay in the study.

CONSEQUENCES OF WITHDRAWAL

You may withdraw your child from the study at any stage if you desire. The study doctors may decide to withdraw your child from the study if they think this is best for your child. In this case, your child will still be eligible for care at Walukuba Health Centre and at other local clinics.

USE OF THE RESULTS

The results from this study may be published in research publications or reported at research meetings. Your child will not be identified by name.

TREATMENT AND COMPENSATION FOR INJURY

If you are injured or have questions about injuries as a result of being in the study, please contact the doctors in the study clinic or Dr. Chi Eziefula (telephone 0784448758) or Dr. Arthur Mpimbaza (telephone 0712 846 903 or 0702 846 903). The sponsoring organisation, the London School of Hygiene & Tropical Medicine, holds insurance policies which apply to this study. If your child experiences harm or injury as a result of taking part in this study, you may be eligible to claim compensation.

QUESTIONS

If you have any other questions about the study, you may call Dr. Chi Eziefula (telephone 0784448758) or Dr. Arthur Mpimbaza (telephone 0712 846 903 or 0702 846 903). You may also contact Dr. Charles Ibingira (telephone 0414-530020) at Mulago Hospital, who approved this study.

WHAT YOUR SIGNATURE OR THUMBPRINT MEANS

Your signature or thumbprint below means that you understand the information given to you about your child's taking part in the study and you agree with the following statements:

"I have read the consent form concerning this study (or have understood the verbal explanation of the consent form) and I understand what will be required of me and what will happen to my child if we take part in it."

"My questions concerning this study have been answered by the person who signed below."

“I agree to results arising from my child’s participation in the study being included in any reports about the study”

"I understand that at any time, I may withdraw from this study without giving a reason and without affecting my normal care and management."

"I agree to my child taking part in this study."

You will also be invited to sign another informed consent forms for the future use of stored specimens.

If you wish your child to participate in this study, you should sign or place your thumbprint below.

WE WILL GIVE YOU A COPY OF THIS SIGNED AND DATED CONSENT FORM

Name of Participant (printed)	Study ID number
Name of Parent/Guardian	
Signature or Fingerprint * of Parent/Guardian	Date
Name of Investigator Administering Consent (printed)	Position/Title
Signature of Investigator Administering Consent	Date
Name of Translator	
Signature of Translator	Date

*If the parent or guardian is unable to read and/or write, an impartial witness should be present during the informed consent discussion. After the written informed consent form is read and explained to the participant and parent or guardian, and after they have orally consented to their child's participation in the trial, and have either signed the consent form or provided their fingerprint, the witness should sign and personally date the consent form. By signing the consent form, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by the parent or guardian, and that informed consent was freely given by the participant and parent or guardian.

Name of Person Witnessing Consent (printed)	
Signature of Person Witnessing Consent	Date

APPENDIX D INFORMED CONSENT FOR FUTURE USE OF BIOLOGICAL SPECIMENS

Appendix D
Informed consent form for future use of biological specimens

Protocol title:	Evaluation of the efficacy and safety of Primaquine for clearance of gametocytes in uncomplicated falciparum malaria
Source of funding:	The Wellcome Trust
Sponsor:	London School of Hygiene and Tropical Medicine
Site of Research:	Walukuba Health Centre IV, Jinja, Uganda
Principle investigator:	Dr Alice C. Eziefula, MBBS MA MRCP MRCPath
Date:	1st September 2011

INTRODUCTION

While your child is in this study, blood samples will be taken from them for tests for this study. Some of these samples may be useful to test other research questions in the future. We are reading you this information to ask if you will donate these samples to be used for medical research in the future. The samples will be stored for a long time in Uganda at Makerere University Medical School and in the United Kingdom at the London School of Hygiene and Tropical Medicine. Samples may also be shared with investigators at other institutions for the purposes of research if they have permission from the study organisers.

It is your choice whether the samples from your child or the child under your care can be used for future research. Take your time to make your decision. If you agree for the samples from your child or the child under your care to be used for future research, we will ask you to sign this consent form. You will get a copy of this form to keep. If at any time you change your mind about the samples from your child/ the child under your care being used for future research, then your decision will be respected and the samples will be disposed of.

WHAT SAMPLES WILL BE USED FOR

The blood samples taken during this study will be used to find the answers to the study question about how primaquine can stop the spread of malaria. After the study, the samples will not be thrown away; they will be kept so they can be used for future research if you decide to give permission.

1. In the future, these samples may be used for research to learn more about malaria and the malaria parasites in your child's blood. If researchers wish to study any other diseases in the future, they will seek permission to use your child's blood samples from the Institutional Review Boards first. The results of these studies will not affect your child's care.

2. Your child's samples will be used only for research and will not be sold or used for the production of commercial products.

3. Whilst in the study, a genetic test will be performed to see if your child has the G6PD (glucose-6-phosphate dehydrogenase) deficiency characteristic. Genetic tests look at the characteristics of a person. The information from these tests will be available to you through the study coordinator, but it will not be kept in your child's medical records. After the study, the stored blood samples may be used for other genetic tests that have to do with how the body responds to malaria. No genetic information obtained from this research will be placed in your child's health centre records. These samples will be identified only by codes so that they cannot be easily identified with your child.

LEVEL OF IDENTIFICATION

Your child's blood samples will be coded with numbers instead of a name so that your child cannot easily be identified. Reports about research done with your child's samples will not be put in their health centre record and will be kept private and safe to the best of our ability.

In the future, researchers studying your child's samples may need to know more about your child, such as their age, gender, and race. If this information is available in your child's study records, it may be provided to the researcher. Your child's name or anything that might identify them personally will not be provided. You will not be asked to provide additional consent.

RISKS

There are few risks to your child from future use of their samples. A potential risk might be the release of information from your child's records. The study records will be kept private and safe as far as possible. Final reports about research done with your child's samples will not be put in their health centre record.

BENEFITS

There will be no direct benefit to your child. From studying your child's samples we may learn more about malaria: how to prevent it, how to treat it, how to cure it.

FINANCIAL ISSUES

If you agree to donate your child's blood samples to medical research, you will not be charged for this and you will not be paid for it. If the research leads to any discoveries that may have a commercial value, your child will not share in any financial benefits.

RESEARCH RESULTS/MEDICAL RECORDS

Results from future research using your child's samples may be presented in research publications and meetings but your child's name will not be identified.

Reports from future research done with your child's samples will not be given to you or your child's doctor. These reports will not be put in your child's medical record.

QUESTIONS

If you have any questions, comments or concerns about the future use of your child's specimen's, first talk to the research staff at the clinic. You may also contact Dr. Chi Eziefula (telephone 0784448758) or Dr. Arthur Mpimbaza (telephone 0712 846 903 or 0702 846 903). If for any reason you do not wish to do this, or you still have concerns about the future use of your child's specimens, you may contact Dr. Charles Ibingira (telephone 0414-530020), Makerere University School of Medicine Research and Ethical Committee.

FREEDOM TO REFUSE

You can change your mind at any time about allowing your child's samples to be used for future research. If you do change your mind, you can contact Dr. Chi Eziefula (telephone 0784448758) or Dr. Arthur Mpimbaza (telephone 0712 846 903 or 0702 846 903). Then your child's samples will no longer be made available for research and we will make all efforts to dispose of the samples. Whether or not you allow us to use your child's samples in future research will not have any effect on your child's participation in this study or future participation in other studies.

WHAT YOUR SIGNATURE OR THUMBPRINT MEANS

Your signature or thumbprint below means that you have had enough time to ask questions and to understand the information given to you in this consent form about your child's specimens to be used

for future research. If you wish to allow your child's specimens to be used for future research, you should sign or place your thumbprint below.

WE WILL GIVE YOU A COPY OF THIS SIGNED AND DATED CONSENT FORM

Name of Participant (printed)	Study ID number
Name of Parent/Guardian	
Signature or Fingerprint * of Parent/Guardian	Date
Name of Investigator Administering Consent (printed)	Position/Title
Signature of Investigator Administering Consent	Date
Name of Translator	
Signature of Translator	Date

*If the parent or guardian is unable to read and/or write, an impartial witness should be present during the informed consent discussion. After the written informed consent form is read and explained to the participant and parent or guardian, and after they have orally consented to their child's participation in the trial, and have either signed the consent form or provided their fingerprint, the witness should sign and personally date the consent form. By signing the consent form, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by the parent or guardian, and that informed consent was freely given by the participant and parent or guardian.

Name of Person Witnessing Consent (printed)	
Signature of Person Witnessing Consent	Date

APPENDIX E ASSENT FORM

Research participant assent form for children

Protocol Title:	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda
Site of Research:	Walukuba Health Centre IV, Jinja, Uganda
Principal Investigators:	Dr. Chi Eziefula
Date:	27 May 2011

- Š I am being asked to decide if I want to be in this research study.
- Š I know that if I accept, I will come to the clinic every day for treatment and then six more times this month
- Š Each time I come to the clinic, the staff will talk to me, ask me questions, and examine me.
- Š I know I will have a few drops of blood drawn from my finger each time I come to the clinic for the study.
- Š I know that they will give me medicine for malaria and an extra tablet for malaria that they are testing
- Š I asked and got answers to my questions. I know that I can ask questions about this survey at any time.
- Š I know that I can stop being in this survey at anytime without anyone being mad at me.

Mark one box with X :

I DO CONSENT: ☐ I hereby agree to take part in this study

I DO NOT CONSENT: ☐ I do not wish to take part in this study

Name of child :	
Signature or fingerprint of child :	Date:

Witness: I hereby confirm that the study has been explained to the child. All questions (if any) have also been answered to his/her satisfaction, and he/she has, of his own free will, consented to take part in the survey.

Name of w itness:	
Signature of w itness:	Date:

Name of person explaining study:	
Signature :	Date:

APPENDIX F SCREENING FORM

SCREENING FORM

SCREENING ID: |____|____|____|____|	**Date of screening:** |____|____|/|____|____|/|____|____| ***day month year***
Initials*	**Date of birth** |____|____|/|____|____|/|____|____| ***day month year***
Age: |____| **years** |____|____| **months**	**Gender:** **Male=0 Female=1** |____|

**Initial of last name, followed by initial of first name*

ASSESS DURING SCREENING INTERVIEW			
Selection criteria	**Include**	**Exclude**	**Code**
1. Age ≥1 year and ≤10 years	Yes=1	No=0	
2. Enrolled in another research study?	No=0	Yes=1	
3. Known or suspected serious chronic illness (eg *AIDS, malnutrition ,cancer)?*	No=0	Yes=1	

4. Intention to move from Walukuba during the study period?	No=0	Yes=1			
5. Taken Primaquine in the 4 weeks prior to the study?	No=0	Yes=1			
6. Taken antimalarials in the 2 days prior to the study?	No=0	Yes=1			
7. History of serious side effects with primaquine or AL?	No=0	Yes=1			
8. Started menstruating?	No=0	Yes=1			
9. Pregnant or breastfeeding?	No=0	Yes=1			
10. Blood transfusion in the last 90 days?	No=0	Yes=1			
If any boxes in the "Exclude" column are ticked, exclude from the study. If not, proceed to the next section.					

INFORMED CONSENT DISCUSSION				
Selection criteria	**Include**	**Exclude**	**Code**	
11. Parents or guardians provided written informed consent?	Yes=1	No=0		
If the box in the "Exclude" column are ticked, exclude from the study. If not, proceed to the next section.				

CLINICAL SCREENING: MEASURE WEIGHT AND TEMPERATURE AND ASSESS MALARIA STATUS				
Selection criteria	**Include**	**Exclude**	**Code**	
12. Weight < 10 kg	No=0	Yes=1		
13. Temperature >38°C or history if fever within 24 hours	Yes=1	No=0		
14. Evidence of severe malaria or danger signs (see SOP for WHO criteria)	No=0	Yes=1		

LABORATORY SCREENING: MEASURE HAEMOGLOBIN				
Selection criteria	**Include**	**Exclude**	**Code**	
15. Haemoglobin < 8.0 g/dL?	No=0	Yes=1		
16. G6PD deficiency?	No=0	Yes=1		

All criteria for study inclusion met?	**Date of enrollment** *(date study begins)*

Yes=1 No=0 Code: |___| (*If no, exclude from the study)*	|___|___|/|___|___|/|___|___| ***day month year***

ASSIGN **STUDY NUMBER**	**PQ-**|____|____|____|____|

APPENDIX G ENROLLMENT FORM

ENROLLMENT **FORM**	**Study Number:** **PQ-** |___|___|___|___|	**Patient Initials:**	**Gender:** |___| **male=0 female=1**
	Today's Date: **|___|___|/|___|___|/|___|___|** ***day month year***	**Age:** |___|___| **years** |___|___| **months** *(include months only if age < 8)*	
Preferred language: |____| **English=0, Lusoga=1, Luganda=2, Swahili=3**			

PAST AND CHRONIC ILLNESSES	
Prior illnesses including blood disorders (include dates, if available) _____ _____ _____ _____ _____	Prior surgeries (include dates, if available) _____ _____ _____ _____
Known drug allergies (include details)	

MEDICATION RECORD (taken in the last 2 weeks)			
Medication	Code	Indication	Date Prescribed

CLINICAL EXAMINATION			
NUTRITIONAL STATUS			
Weight (kg) |___|___|	*Height (cm)* |___|___|___|	*HAZ -score (circle + or -):* + / - _ B B B _ ǂ _ B B B _	*WHZ -score (circle + or -):* + / - _ B B B _ ǂ _ B

LABORATORY TESTS		
	Result	Staff Initials
P. falciparum parasite density (/ul)		
Gametocytes (Y/N)		
Hemoglobin (g/dL)		
‘Enrolled Date of enrollment: |___|___|/|___|___|/|___|___| day month year		
‘Excluded Date of exclusion: |___|___|/|___|___|/|___|___|		

APPENDIX H WEIGHT-BASED TREATMENT GUIDELINES (AL)

The dosing schedule for artemether-lumefantrine in this study is according to manufacturer and Ugandan national malaria control programme guidelines, as follows:

Weight (Kg)	Age	Tablets per Treatment Course	Color Code*
5–14	From 4 months to 3 years	6 x 1 tablets	Yellow
15–24	From 3 years to 7 years	6 x 2 tablets	Blue
25–34	From 7 years to 12 years	6 x 3 tablets	Brown
> 35	12 years and above	6 x 4 tablets	Green

*Color codes correspond to the pack sizes for the different age groups.

Each dose (1-4 tablets) is given twice daily for three days. Weight rather than age criteria will be used.

APPENDIX I DOSING OF STUDY DRUG (PRIMAQUINE)

	Primaquine: number of mg given on day 2							
PQ dose	**Placebo (0 mg/kg)**		**0.1mg/kg**		**0.4mg/kg**		**0.75mg/kg**	
Wt (Kg)	**Target dose**	**Actual dose**	**Target dose**	**Actual dose**	**Target dose**	**Actual dose**	**Target dose**	**Actual dose**
10	0	0	1	1	4	4	7.5	7.5
11	0	0	1.1	1	4.4	4	8.25	8
12	0	0	1.2	1	4.8	4	9	9
13	0	0	1.3	1	5.2	5	9.75	9.5
14	0	0	1.4	1	5.6	5.5	10.5	10
15	0	0	1.5	1.5	6	6	11.25	11
16	0	0	1.6	1.5	6.4	6	12	12
17	0	0	1.7	1.5	6.8	6.5	12.75	12.5
18	0	0	1.8	1.5	7.2	7	13.5	13.5
19	0	0	1.9	1.5	7.6	7.5	14.25	14
20	0	0	2	2	8	8	15	15

21	0	0	2.1	2	8.4	8	15.75	15.5
22	0	0	2.2	2	8.8	8.5	16.5	16
23	0	0	2.3	2	9.2	9	17.25	17.5
24	0	0	2.4	2	9.6	9.5	18	18
25	0	0	2.5	2.5	10	10	18.75	18.5
26	0	0	2.6	2.5	10.4	10	19.5	19.5
27	0	0	2.7	2.5	10.8	10.5	20.25	20
28	0	0	1	1	11.2	11	21	21
29	0	0	1.1	1	11.6	11.5	21.75	21.5
30	0	0	1.2	1	12	12	22.5	22.5
31	0	0	1.3	1	12.4	12	23.25	23
32	0	0	1.4	1	12.8	12.5	24	24
33	0	0	1.5	1.5	13.2	13	24.75	24.5
34	0	0	1.6	1.5	13.6	13.5	25.5	25
35	0	0	1.7	1.5	14	14	26.25	26
36	0	0	1.8	1.5	14.4	14	27	27
37	0	0	1.9	1.5	14.8	14.5	27.75	27.5
38	0	0	2	2	15.2	15	28.5	28.5
39	0	0	2.1	2	15.6	15.5	29.25	29
40	0	0	2.2	2	16	16	30	30

STUDY NUMBER:_____

APPENDIX J CLINIC CARD –STUDY DRUG VISITS

Clinic card

Day 0 Date:

Doctor station	Seen by (initials)	

Nurse station	Seen by (initials)	Number of tablets	Number of mls	Vomited first dose?	Vomited second dose?	Vomited third dose?
1st AL dose						
2nd AL dose						

Laboratory station	Seen by (initials)	Finger prick taken

Day 1 Date:

Doctor station	Seen by (initials)	

Nurse station	Seen by (initials)	Number of tablets	Number of mls	Vomited first dose?	Vomited second dose?	Vomited third dose?
1st AL dose						
2nd AL dose						

Laboratory station	Seen by (initials)	Finger prick taken

Day 2 Date:

Doctor station	Seen by (initials)	

Nurse station	Seen by (initials)	Number of tablets	Number of mls	Vomited first dose?	Vomited second dose?	Vomited third dose?
1st AL dose						
PQ/ placebo						
2nd AL dose						

Laboratory station	Seen by (initials)	Finger prick taken

APPENDIX K HOUSEHOLD CONTACT FORM

HOUSEHOLD CONTACT FORM

CONFIDENTIAL INFORMATION: COMPLETE FORM AND FILE SEPARATELY

Participant Initials:	**Study Number:** **PQ-**|___|___|___||___|	**Gender:** |___| Male=0, Female=2	**Household GPS reading:** |___|___|___|___|___|___|___|___|
Date of enrollment: |___|___|/|___|___|/|___|___| ***day month year***		**Age:_____years_____months** *(include months only if age < 5)*	

Participant's name *(last, first):*	
Head of household's name:	
Relationship to participant:	
Primary caregiver's name:	
Relationship to participant:	
Father's name:	
Mother's name:	
Name of other household member enrolled in this study:	Study Number: **PQ-**|___|___|___||___|

Name of other household member enrolled in this study:	Study Number: **PQ-**|___|___|___||___|
Name of other household member enrolled in this study:	Study Number: **PQ-**|___|___|___||___|
Name of other household member enrolled in this study:	Study Number: **PQ-**|___|___|___||___|
Name of other household member enrolled in this study:	Study Number: **PQ-**|___|___|___||___|
Name of other household member enrolled in this study:	Study Number: **PQ-**|___|___|___||___|
Home parish:	LC1:
Home address if available:	
Phone number: No=0 Yes=1 Unknown=2 Code: |___| If yes: Number 1: _____ Name of phone owner: _____ Number 2: _____ Name of phone owner: _____	

APPENDIX L CASE RECORD FORMS

CLINICAL RECORD FORM (1) ***HISTORY***	**Patient Initials:**	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***day month year***	**Study Number: PQ-** |___|___|___|___|

SYMPTOM RECORD

(Rank on scale of 0-4: absent = 0; mild = 1; moderate = 2; severe = 3, life-threatening = 4, N/A = unable to assess)

	DAY 0	**DAY 1**	**DAY 2**	**DAY 3**	**DAY 7**	**DAY 10**	**DAY 14**	**DAY 21**	**DAY 28**
DATE									
SYMPTOMS									
Fever *(Y/N)* [grade]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
Weakness									
Headache†									
Anorexia									
Nausea†									
Vomiting									
Abdominal pain†									
Diarrhea									
Cough									
Pruritis									
Joint pains									
Urine colour									
Other_____									
Other_____									

CLINICAL HISTORY RECORD

Record clinically relevant details of history									
Initials									

CLINICAL RECORD FORM (2) ***EXAMINATION***	**Patient Initials:**	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***day month year***	**Study Number: PQ-** |___|___|___|___|

PHYSICAL EXAM RECORD

(Rank on scale of 0-4: absent = 0; mild = 1; moderate = 2; severe = 3, life-threatening = 4, N/A = unable to assess)

	DAY 0	**DAY 1**	**DAY 2**	**DAY 3**	**DAY 7**	**DAY 10**	**DAY 14**	**DAY 21**	**DAY 28**
DATE									
PHYSICAL EXAM									
Temperature *(ºC)* [grade]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
Respiratory rate									
Pallor									
Jaundice									
Eyes									

Oropharynx									
Neck									
Chest									
CVS									
Abdomen									
Skin									
Hearing									
Tablet test/ CNS/ PNS									
Urine (state colour)									
Other_____									
Other_____									
ABNORMAL EXAM RECORD									
If abnormality noted on physical exam,, describe all physical findings for the abnormal exam									
Initials									

APPENDIX M UNSCHEDULED VISITS

CLINICAL RECORD FORM (3) ***UNSCHEDULED VISITS: HISTORY***	**Patient Initials:**	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***daymonth year***	**Study Number: PQ-** |___|___|___|___|
SYMPTOM RECORD ***(Rank on scale of 0-4: absent = 0; mild = 1; moderate = 2; severe = 3, life-threatening = 4, N/A = unable to assess)***			

	DAY __	DAY __	DAY __	DAY __	DAY __	DAY __	DAY __	DAY __	DAY __
DATE									
Reason for unscheduled visit									
SYMPTOMS									
Fever *(Y/N)* [grade]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
Weakness									
Headache†									
Anorexia									
Nausea†									
Vomiting									
Abdominal pain†									
Diarrhea									
Cough									
Pruritis									
Joint pains									
Urine colour									
Other_____									
Other_____									
CLINICAL HISTORY RECORD									

Record clinically relevant details of history									
Initials									

CLINICAL RECORD FORM (3) ***UNSCHEDULED VISITS: EXAM***	**Patient Initials:**	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***day month year***	**Study Number: PQ-** |___|___|___|___|

PHYSICAL EXAM RECORD

(Rank on scale of 0-4: absent = 0; mild = 1; moderate = 2; severe = 3, life-threatening = 4, N/A = unable to assess)

	DAY __	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**
DATE									
PHYSICAL EXAM									
Temperature *(ºC)* [grade]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
Respiratory rate									
Pallor									
Jaundice									
Eyes									
Oropharynx									

Neck									
Chest									
CVS									
Abdomen									
Skin									
Hearing									
Tablet test/ CNS/ PNS									
Urine (state colour)									
Other_____									
Other_____									
ABNORMAL EXAM RECORD									
If abnormality noted on physical exam,, describe all physical findings for the abnormal exam									
Initials									

CLINICAL RECORD FORM (3) ***UNSCHEDULED VISITS: MANAGEMENT***	**Patient Initials:**	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***day month year***

Study Number: PQ-
|___|___|___|___|

	DAY __	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**	**DAY __**
DATE									

Management summary for unscheduled visit									
Initials									

APPENDIX N HOSPITAL FOLLOW UP RECORD

HOSPITAL ADMISSION FORM		
Patient initials:	**Study Number: PQ-**|___|___|___|___|	**Age:_____years_____months**

Date of admission : |___|___|/|___|___|/|___|___| *day month year*	Study Day:
Date of discharge* : |___|___|/|___|___|/|___|___| *day month year*	Study Day:

Reason for admission:	
History:	Exam:
Laboratory Results (with dates samples taken):	

Assessment/Plan: *(List all medications given during hospitalization on FOLLOW UP FORMS)*

**** Record date when patient has been discharged from the hospital***

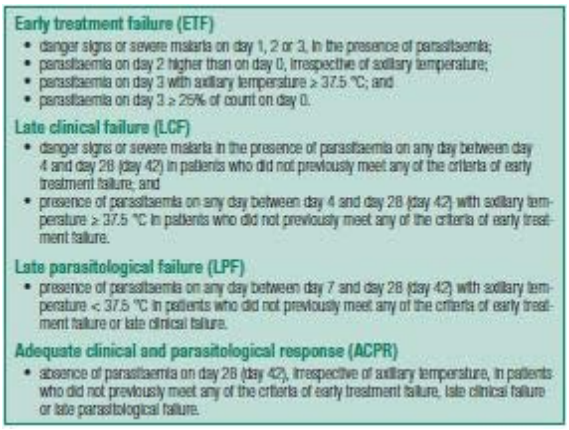
HOSPITAL FOLLOW-UP FORM

Patient initials:	**Study Number: PQ-** |___|___|___|___|	**Admit Date:** |___|___|/|___|___|/|___|___| ***day month year***

Date of follow-up: |___|___|/|___|___|/|___|___| *day month year*	Study Day:	Temp:
Progress Note: *Initials:_____*		
Date of follow-up: |___|___|/|___|___|/|___|___| *day month year*	Study Day:	Temp:

Progress Note: *Initials:_____*		
Date of follow-up: |___|___|/|___|___|/|___|___| *day month year*	Study Day:	Temp:
Progress Note: *Initials:_____*		

APPENDIX O METHODS FOR SURVEILLANCE OF ANTIMALARIAL DRUG EFFICACY (WHO, 2009)



Early treatment failure (ETF)

- danger signs or severe malaria on day 1, 2 or 3, in the presence of parasitaemia;
- parasitaemia on day 2 higher than on day 0, irrespective of axillary temperature;
- parasitaemia on day 3 with axillary temperature ≥ 37.5 °C; and
- parasitaemia on day 3 ≥ 25% of count on day 0.

Late clinical failure (LCF)

- danger signs or severe malaria in the presence of parasitaemia on any day between day 4 and day 28 (day 42) in patients who did not previously meet any of the criteria of early treatment failure; and
- presence of parasitaemia on any day between day 4 and day 28 (day 42) with axillary temperature ≥ 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure.

Late parasitological failure (LPF)

- presence of parasitaemia on any day between day 7 and day 28 (day 42) with axillary temperature < 37.5 °C in patients who did not previously meet any of the criteria of early treatment failure or late clinical failure.

Adequate clinical and parasitological response (ACPR)

- absence of parasitaemia on day 28 (day 42), irrespective of axillary temperature, in patients who did not previously meet any of the criteria of early treatment failure, late clinical failure or late parasitological failure.

APPENDIX P INFORMED CONSENT FOR PHARMACOKINETIC STUDY

Appendix P
Informed consent form for participation in pharmacokinetic research

Protocol title: Evaluation of the efficacy and safety of Primaquine for clearance of gametocytes in uncomplicated falciparum malaria

Source of funding:	The Wellcome Trust
Sponsor:	London School of Hygiene and Tropical Medicine
Site of Research:	Walukuba Health Centre IV, Jinja, Uganda
Principle investigator:	Dr Alice C. Eziefula, MBBS MA MRCP MRCPath
Date:	1st September 2011

PURPOSE OF THE STUDY

You are being asked whether you will let your child/ the child under your care participate part in an extra study which is part of the main study you are enrolled in. This involves some extra blood tests over the next three days.

This information is being read to you in order to give you information about this extra study so that you know what will be involved. We will explain the purpose of this study, how the study will be done, and any risks and benefits. You can ask questions at any time. After this consent form is read to you, and your questions have been answered, you will decide if your child or the child under your care will participate in the study. Medical research includes only people who choose to take part. So it is your choice whether your child or the child under your care will take part. Take your time to make your decision about participating. If you agree for your child or the child under your care to participate in this study, we will ask you to sign this consent form. You will get a copy of this form to keep. If at any time you change your mind about your child/ the child under your care participating in the study, then your decision will be respected.

This study is being done by researchers from universities in Uganda (Makerere University and the Uganda Malaria Surveillance Project) and the United Kingdom (London School of Hygiene and Tropical Medicine) and Thailand (Mahidol Oxford Research Unit).

The purpose of this study is to learn more about how the study drug, primaquine works in different people. Some drugs work differently in males and females and in people of different ages. We need more information about this drug in Ugandan children in order to be sure that it has the correct effect.

HOW THE STUDY IS DONE

The pharmacokinetic study will involve:

-coming early to the clinic (by 07.30) on the next three days

-staying in the study clinic between 08.00 hours and 05.00 hours tomorrow

-having extra blood samples taken over the next three days

This is explained in detail as follows:

You will be asked to come early with your child to the study clinic tomorrow, by 07.30 hours. On arrival at the clinic, your child will be seen by a doctor and have a plastic tube (venous canula) fixed in their arm so that blood samples can be taken. This is so that the rest of the blood samples that day can be taken without the need to pierce the child's skin again.

Your child will then be given their study drugs. You and your child will need to stay in the study clinic for the rest of the day. During this time, the doctor will take four more blood samples from your child at fixed times.

After the last sample tomorrow, the plastic tube will be removed from your child's arm and you can go home.

The next two days, you are asked to come back to the study clinic at 07.30 hours. On each day your child will be seen by the doctor and one blood sample will be taken, after which you can go home.

The blood samples will be sent to Thailand to get the results. They will be kept there until we are allowed to close the study. They may be used for further research on malaria in Thailand after the study, but your child's name will not be easily identified. The blood samples will be identified with a code.

DURATION OF PARTICIPATION IN THE PHARMACOKINETIC STUDY

This section of the study lasts three days. It requires that you attend the clinic early in the next three days for extra blood tests. After that, the study staff will ask you to come back to the clinic according to the appointments on your card for the main study.

PROCEDURES

RISKS AND DISCOMFORTS

Blood tests: The risks of drawing blood include temporary discomfort from the needle point, bruising, and skin infection. The amount of blood removed will be too small to affect your child's health.

Confidentiality: Taking part in the study may involve a loss of privacy, because we will collect information about your child's health in a record on paper and on a computer, but information about your child will be handled as confidentially as possible. Only the people working on the study and researchers with permission will see it. During the study period, these records will be kept at the Walukuba study office and the IDRC offices at Mulago Hospital. Dr. Eziefula will be responsible for the

use, storage, and disposal of records. Records will be kept as private as possible. People responsible for making sure that the research is done properly may ask to see your child's records. If you sign this consent form, you are allowing your child's records to be seen by these people.

BENEFITS

All children taking part in the study will have their malaria infection treated with the best treatment available, in accordance with the Ugandan national guidelines. There is no personal immediate benefit to your child from the study medication (primaquine). The medication is given to reduce the chance that that your child can pass malaria on to a mosquito, reducing the risk of that mosquito passing on malaria to other people at home or in the local area.

The results of the study may be used to decide if in the future, primaquine might be used to reduce malaria in the community.

COST/ PAYMENT

You will not be charged for any of the study treatments or procedures. You will be responsible for all costs for your child's routine health care. You and your child will not be paid for participation in the study.

The cost of transport of your child to and from the study clinic for follow up visits or for any extra visits during the time your child is in the study will be reimbursed to you. The sum of 7000/- UGX (equivalent to $2.60) will be given at each clinic visit to cover the journey home and the next return visit from home to the clinic.

ALTERNATIVES TO PARTICIPATION

Participation in this study is completely voluntary. If you decide you do not want yourself or your child to participate in the study, this will not affect your child’s care at local clinics or at Jinja Hospital or Walukuba Health Centre. During the study, you will be informed promptly of any new information that may influence your willingness to continue participation in the study.

CONSEQUENCES OF WITHDRAWAL

You may withdraw your child from the study at any stage if you desire. The study doctors may decide to withdraw your child from the study if they think this is best for your child. In this case, your child will still be eligible for care at Walukuba Health Centre and at other local clinics.

USE OF THE RESULTS

The findings from this study may be published in research publications or reported at research meetings. Your child will not be identified by name.

TREATMENT AND COMPENSATION FOR INJURY

If you are injured or have questions about injuries as a result of being in the study, please contact the doctors in the study clinic or Dr. Chi Eziefula (telephone 0784448758) or Dr. Arthur Mpimbaza (telephone 0712 846 903 or 0702 846 903). The sponsoring organisation, the London School of Hygiene & Tropical Medicine, holds insurance policies which apply to this study. If your child experiences harm or injury as a result of taking part in this study, you may be eligible to claim compensation

QUESTIONS

If you have any other questions about the study, you may call Dr. Chi Eziefula (telephone 0784448758) or Dr. Arthur Mpimbaza (telephone 0712 846 903 or 0702 846 903). You may also contact Dr. Charles Ibingira (telephone 0414-530020) at Mulago Hospital, who approved this study.

WHAT YOUR SIGNATURE OR THUMBPRINT MEANS

Your signature or thumbprint below means that you understand the information given to you about your child's participation in the study and in this consent form and agree with the following statements:

"I have read the consent form concerning this study (or have understood the verbal explanation of the consent form) and I understand what will be required of me and what will happen to my child if we take part in it."

"My questions concerning this study have been answered by the person who signed below."

"I agree to results arising from my participation in the study being included, even anonymously in any reports about the study"

"I understand that at any time, I may withdraw from this study without giving a reason and without affecting my normal care and management."

"I agree to my child taking part in this study."

If you wish your child to participate in this study, you should sign or place your thumbprint below.

WE WILL GIVE YOU A COPY OF THIS SIGNED AND DATED CONSENT FORM

Name of Participant (printed)	Study ID number
Name of Parent/Guardian	
Signature or Fingerprint * of Parent/Guardian	Date
Name of Investigator Administering Consent (printed)	Position/Title
Signature of Investigator Administering Consent	Date
Name of Translator	
Signature of Translator	Date

*If the parent or guardian is unable to read and/or write, an impartial witness should be present during the informed consent discussion. After the written informed consent form is read and explained to the participant and parent or guardian, and after they have orally consented to their child's participation in the trial, and have either signed the consent form or provided their fingerprint, the witness should sign and personally date the consent form. By signing the consent form, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by the parent or guardian, and that informed consent was freely given by the participant and parent or guardian.

Name of Person Witnessing Consent (printed)

Signature of Person Witnessing Consent Date

Pharmacokinetics assent form for children

Protocol Title:	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda
Site of Research:	Walukuba Health Centre IV, Jinja, Uganda
Principal Investigators:	Dr. Chi Eziefula
Date:	27 May 2011

Š I am being asked to decide if I want to be in a study to measure the amount of medicines in my blood Š I know that if I accept, I will have seven extra blood tests in the next three days Š I know that I will have a canula fitted tomorrow to take blood samples

Š I asked and got answers to my questions. I know that I can ask questions about this survey at any time.

Š I know that I can stop being in this survey at anytime without anyone being mad at me.

Mark one box with X :

I DO CONSENT: ☐ I hereby agree to take part in this study

I DO NOT CONSENT: ☐ I do not wish to take part in this study

Name of child :	
Signature or fingerprint of child :	Date:

Witness: I hereby confirm that the study has been explained to the child. All questions (if any) have also been answered to his/her satisfaction, and he/she has, of his own free will, consented to take part in the survey.

Name of w itness:

Signature of w itness:	Date:

Name of person explaining study:	
Signature :	Date:

APPENDIX Q ADVERSE EVENT REPORTING FORM

SERIOUS adverse event form – Initial report	**UNCST study number:**	
Study Number: **PQ-** |___|___|___|___|	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***day month year***	**Gender** |___| Male=0, Female=1

Event description:_____

(symptom, sign, or laboratory abnormality)

Date of event onset: |___|___|/|___|___|/|___|___|___|___| day month year	Date event reported: |___|___|/|___|___|/|___|___|___|___| day month year	Indicate reason for serious AE: Fatal Life-threatening Resulted in significant /persistent disability or incapacity Resulted in hospitalization Prolonged hospitalization Required medical / surgical intervention to prevent serious outcome Other:_____
Maximum event severity: Mild Moderate Severe Life-threatening	Maximum relationship to study drugs: None Unlikely Possible Probable Definite	
Was the event unexpected? Expected=0 Unexpected=1 |___|		

Event history (symptoms, signs, differential diagnoses):	Event history (medical management): Relevant past medical history:

Concomitant medications taken in the last month:

Medication	Start date (dd/mm/yy)	Stop date (dd/mm/yy)	Total daily dose	Indication	Suspect for SAE? N=0, Y=1
	|_|_|/|_|_|/|_|_|	|_|_|/|_|_|/|_|_|			|___|
	|_|_|/|_|_|/|_|_|	|_|_|/|_|_|/|_|_|			|___|
	|_|_|/|_|_|/|_|_|	|_|_|/|_|_|/|_|_|			|___|
	|_|_|/|_|_|/|_|_|	|_|_|/|_|_|/|_|_|			|___|
	|_|_|/|_|_|/|_|_|	|_|_|/|_|_|/|_|_|			|___|
	|_|_|/|_|_|/|_|_|	|_|_|/|_|_|/|_|_|			|___|
	|_|_|/|_|_|/|_|_|	|_|_|/|_|_|/|_|_|			|___|

Date form completed: |___|___|/|___|___|/|___|___|___|___| *day month year*	Investigator's name (printed): _____ Investigator's signature: _____

SERIOUS adverse event form – Initial report	**UNCST study number:**	
Study Number: **PQ-** |___|___|___|___|	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***day month year***	**Gender** |___| Male=0, Female=1

Event description:_____

(symptom, sign, or laboratory abnormality)

Date of event onset:	Date event reported:
|___|___|/|___|___|/|___|___|___|___|	|___|___|/|___|___|/|___|___|___|___|
day month year	day month year

Relevant diagnostic tests:

Test	Collection date (dd/mm/yy)	Result	Normal range	Most recent value prior to SAE	Collection date (recent value) (dd/mm/yy)
	|__|__|/|__|__|/|__|__|				|__|__|/|__|__|/|__|__|
	|__|__|/|__|__|/|__|__|				|__|__|/|__|__|/|__|__|
	|__|__|/|__|__|/|__|__|				|__|__|/|__|__|/|__|__|
	|__|__|/|__|__|/|__|__|				|__|__|/|__|__|/|__|__|
	|__|__|/|__|__|/|__|__|				|__|__|/|__|__|/|__|__|
	|__|__|/|__|__|/|__|__|				|__|__|/|__|__|/|__|__|
	|__|__|/|__|__|/|__|__|				|__|__|/|__|__|/|__|__|

Other diagnostic investigations

Investigation	Date performed	Result

Summary of action taken: (tick all that apply)

No change in current management
Study medication discontinued
Specific treatment given
Patient hospitalized
Laboratory tests obtained
Other:_____
Other:_____

Outcome of event:	If resolved or died, indicate date:
Ongoing (SAE follow up form to be completed and sent at a later date) Resolved without sequelae Resolved with sequelae_____ Death	|___|___|/|___|___|/|___|___|___|___| *day month year*

Date form completed:	Investigator's name (printed): _____
|___|___|/|___|___|/|___|___|___|___| *day month year*	Investigator's signature: _____

SERIOUS adverse event– Follow up report		**UNCST study number:**
Study Number: **PQ-** |___|___|___|___|	**Day 0 Date:** |___|___|/|___|___|/|___|___| ***day month year***	**Gender** |___| Male=0, Female=1

Event description:_____

(symptom, sign, or laboratory abnormality)

Date of first report:	Date of this progress report:
|___|___|/|___|___|/|___|___|___|___| day month year	|___|___|/|___|___|/|___|___|___|___| day month year

Relevant diagnostic tests:

Test	Date collected (dd/mm/yy)	Result	Other investigations or procedures	Date performed (dd/mm/yy)	Result
	|_|_|/|_|_|/|_|_|			|_|_|/|_|_|/|_|_|	
	|_|_|/|_|_|/|_|_|			|_|_|/|_|_|/|_|_|	
	|_|_|/|_|_|/|_|_|			|_|_|/|_|_|/|_|_|	
	|_|_|/|_|_|/|_|_|			|_|_|/|_|_|/|_|_|	
	|_|_|/|_|_|/|_|_|			|_|_|/|_|_|/|_|_|	
	|_|_|/|_|_|/|_|_|			|_|_|/|_|_|/|_|_|	

Progress notes:

Outcome of event:

Ongoing (SAE follow up form to be completed and sent at a later date)

Resolved without sequelae

Resolved with sequelae_____

Death

If resolved or died, indicate date:

|___|___|/|___|___|/|___|___|___|___|

day *month* *year*

Date form completed:

|___|___|/|___|___|/|___|___|___|___|

day *month* *year*

Investigator's name (printed): _____

Investigator's signature: _____

APPENDIX R ADVERSE EVENT GRADING SCHEME

Grade	Severity	Description
1	Mild	Transient or mild discomfort; no limitation in activity; no medical intervention/therapy required
2	Moderate	Mild to moderate limitation in activity – some assistance may be needed; no or minimal medical intervention required
3	Severe	Marked limitation in activity; some assistance usually required; hospitalization possible
4	Life-threatening	Extreme limitation in activity, significant assistance required; significant medical intervention/therapy required; hospitalization probable
5	Death	

APPENDIX S CAUSAL ASSOCIATION OF ADVERSE EVENT WITH USE OF STUDY MEDICATION

Causal relationships of adverse events to anti-malarial agents*

Classification	Definition
Definite	Clear-cut temporal association, with laboratory confirmation, if indicated
Probable	Clear-cut temporal association, with improvement upon study agent withdrawal, and not reasonably explained by the subject's known clinical state
Possible	Less clear temporal association, other etiologies possible
Unlikely	Less clear temporal association; relationship to study agent in doubt
None	Clearly related to other etiologies such as motor vehicle accident

*Adapted from IDRC publications[41]

The following criteria will be assessed in order to establish the suspected relationship of the event to the study medications:

- Expectedness of the event
- Timing of the onset of the event
- New event vs. worsening of a condition present at baseline
- Overall medical condition of the patient, including status of malaria.

APPENDIX B

1) *Clinical Trial Licence Application to Ugandan National Drug Authority*
2) *Investigator's Brochure*

CLINICAL TRIAL APPLICATION FORM

Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda

Prinicpal Investigator:
Dr Chi Eziefula
IDRC, Mulago Hospital Complex, Po Box 7475, Kampala, Uganda

National Drug Authority Plot 48 Lumumba Avenue
Secretariat Office: Plot 46-48 Lumumba Avenue

National Drug Authority
P.O. Box 23096, Kampala, Uganda

Tel: +256-41-255665/347391/347392
Email: ndaug@nda.or.ug
Website: http:// www.nda.or.ug

NATIONAL DRUG AUTHORITY
Clinical trial Application Form

Contents

National Drug Authority Plot 48 Lumumba Avenue
Secretariat Office: Plot 46-48 Lumumba Avenue

National Drug Authority
P.O. Box 23096, Kampala, Uganda

Tel: +256-41-255665/347391/347392
Email: ndaug@nda.or.ug
Website: http:// www.nda.or.ug

Fees; Proof of payment ($1000)

National Drug Authority Plot 48 Lumumba Avenue
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NATIONAL DRUG AUTHORITY
Clinical trial Application Form

Section 1 Identification of the Clinical Trial

1.1 Title of the Study

Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda

1.2 Protocol version number and date

Version number 1.0, date 1st June 2011

1.2 Contact Person

Dr Chi Eziefula
IDRC, Mulago Hospital Complex, Po Box 7475, Kampala, Uganda
Telephone: +256784448758
E-mail: chi.eziefula@gmail.com, chi.eziefula@lshtm.ac.uk

1.4 [Space for NDA Reference Number]

1.5 Declaration of Intent signed by the Principal Investigator

We, the undersigned have submitted all the required documentation and have disclosed all the information required for approval of this application.

We have read the Protocol and the Investigators brochure, appended.
We have the authority and responsibility to oversee this clinical trial, and agree to ensure that the trial will be conducted according to the Protocol and all legal, ethical and regulatory requirements in Uganda.

Applicant (Local Contact): NAME……
Date:

Signature:----------
Designation----------

Principal Investigator: NAME: Dr Chi Eziefula
Date:

Signature:……
Designation….....Clinical Research Fellow……

	Name:	Telephone Number/s:	Fax	E-mail address	Physical Address	Postal address
Applicant	Chi Eziefula	+256784448758	none	chi.eziefula@gmail.com, chi.eziefula@lshtm.ac.uk	Jinja, Uganda	IDRC, Mulago Hospital Complex, Po Box 7475, Kampala Uganda
Sponsor	London School of Hygiene and Tropical Medicine	+44 (0)20 7299 4684	+44 (0)20 7299 4663	patricia.henley@lshtm.ac.uk	Keppel Street, London, WC1E 7HT, UK	Keppel Street, London, WC1E 7HT, UK
Manufacturer	Government pharmaceutical Organization	+662 354 1395	+662 354 9169	Phung@tropmedres.ac	Wellcome Trust Mahidol Oxford Tropical Medicine Research Unit, 420 6 Rajvithi Road, Bangkok, Thailand 10400	Wellcome Trust Mahidol Oxford Tropical Medicine Research Unit, 420 6 Rajvithi Road, Bangkok, Thailand 10400

Note: This is an investigator-led trial sponsored by the applicant's academic institution.

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Secretariat Office: Plot 46-48 Lumumba Avenue

National Drug Authority
P.O. Box 23096, Kampala, Uganda

Tel: +256-41-255665/347391/347392
Email: ndaug@nda.or.ug
Website: http:// www.nda.or.ug

Section 2 Basic Administrative Data on the Application

2.1 Name and address of the registered office of the Applicant

Infectious Diseases Research Collaboration (IDRC), Mulago Hospital Complex, Po Box 7475, Kampala, Uganda

Section 3 Medicines to be used in the trial

3.1 Investigational medicine

3.1.1 Identifier or name of investigational medicine (code if applicable)

Primaquine Phosphate

3.1.2 Registration number

NDA registration number: 1256/06/97

3.1.3 Manufacturer/s (Include all sites)

Government Pharmaceutical Organisation, 75/1 Rama VI Road, Ratchathewi, Bangkok 10400, Thailand
Designated contact: Kanchana Pongsaswat, E-mail: Phung@tropmedres.ac, Wellcome Trust Mahidol Oxford Tropical Medicine Research Unit, 420 6 Rajvithi Road, Bangkok, Thailand 10400

3.1.4 Active ingredient, complete composition, potency and presentation

Each tablet contains 26.3 mg of Primaquine phosphate (equivalent to 15 mg of primaquine base). The dosage is expressed in terms of the base.
Chemical name: 8-[(4-Amino-1-methylbutyl)amino]-6-methoxyquinoline phosphate
Presentation: Dark brown, circular biconvex film-coated tablets

3.1.5 Evidence of manufacture under conditions compliant with current codes of Good Manufacturing Practice

The tablets are manufactured according to the quality assurance standards of the Government Pharmaceutical Organization of Thailand. The manufacturing process is run according to GMP. Please see Appendix 4 for further details.

3.1.6 Release Specifications and tests. Include Certificate of Analysis.

Please see data provided by the manufacturer in Appendix 4.

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3.1.7 Current approved Package Insert if available.
Package insert not available from GPO. The Sanofi-aventis package insert for primaquine phosphate is provided in Appendix 5.

3.2 Comparator, Concomitant and Rescue medications (and Placebo)
3.2.1 Proprietary name and INN
Artemether-lumefantrine

3.2.2 Active ingredient/s, composition, and presentation Artemether-lumefantrine tablets are a fixed dose combination of artemether and lumefantrine in the ratio of 1:6

Each tablet contains 20 mg of artemether and 120 mg lumefantrine
The chemical name of artemether is (3R,5aS,6R,8aS,9R,10S,12R,12aR)-decahydro-10-methoxy-3,6,9-trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-1,2-benzodioxepine

The chemical name of lumefantrine is (±)-2-dibutylamino-1-[2,7-dichloro-9-(4-chlorobenzylidene)-9H-fluorene-4-yl]ethanol

Presentation: yellow, round flat tablets.

3.2.3 Registration number/s (country)
The Ugandan NDA registration number is: 5360/06/06

3.2.4 Approved Package inserts to be appended to application [Appendix 5]
Please see Appendix 5.

3.2.5 Evidence that Placebo is manufactured under GMP. [Appendix 6]
Please see Appendix 6.

3.3 Details of handling Trial medicines
3.3.1 Shipping, delivery and distribution of trial medicines
Government Pharmaceutical Organisation, Thailand will supply the primaquine for the duration of the trial.

3.3.2 Details of storage requirements and arrangements for cold-chain maintenance where necessary and monitoring during distribution.
Storage requirements: Primaquine phosphate tablets should be stored in well-closed, light - resistant containers at a temperature less than 40 deg C, preferably between 15-30 deg C.

3.3.3 Details of dispensing trial medicines and waste disposal procedures. The trial is randomized and double blinded. On days 0-2, artemether lumefantrine is given. Study medication is given on day 2. The study pharmacist will possess the

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assignment code breaker and will dispense the relevant treatment for days 0 -2. The treatment assignment code corresponds to a primaquine/ placebo dose to be given on day 2: this is either placebo or variable dose primaquine. The study pharmacist has access to the code but the study nurses and clinicians do not.

Having selected an opaque envelope for the child, the study nurse will bring the envelope to the study pharmacist. The study pharmacist will open the envelope, document the treatment assignment code and the participant's study number on the treatment assignment log, calculate the correct dose of primaquine/ placebo in milligrams and document the number of millilitres of primaquine/ placebo solution that are required. The treatment assignment code and the dose to be given will not be documented on the CRF or provided to the study nurse.

The procedures for the administration of primaquine/ placebo are as follows:

- At the same time as the fifth dose of AL, in the morning of Day 2, the study nurse requests the primaquine dose from pharmacy.
- The primaquine solution (1mg/ml) is prepared by the study pharmacist by dissolving the primaquine tablets according to a standardized SOP. The study pharmacist documents the dose on the treatment allocation form (as above). The pharmacist draws up the dose into a sterile syringe and hands the syringe to the study nurse.
- The placebo solution is prepared by the study pharmacist by dissolving the placebo tablets according to a standardized SOP. The study pharmacist draws up the pre-determined volume into a sterile syringe and hands the syringe to the study nurse.
- The study nurse administers the liquid primaquine / placebo to the participant on a spoon. The study nurse documents that the primaquine / placebo has been given on the participant's medication record and clinic card.
- The study nurse observes the participant for 30 minutes. Any participant who vomits the medication within 30 minutes of administration will be re-treated with a second dose (requested from pharmacy). Any participant who vomits the primaquine/ placebo dose repeatedly (>3 times) will be excluded from the study. If there is a possibility that they have ingested any of the primaquine dose, they will be excluded from efficacy analysis, but followed up for safety outcomes and adverse events. If the participant vomits, the study nurse documents this on the participant's medication record and clinic card.

The medications used in the study will be supplied to the main study office at the IDRC in Mulago Hospital Complex, Kampala. Artemether-lumefantrine will be ordered through

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the Ugandan Novartis representative, Surgipharm (Kampala, Uganda). Primaquine is ordered through the Government Pharmaceutical Organisation, Bangkok, Thailand. The medications will be stored as per manufacturers' guidelines. Product inserts and detailed documentation relevant to the procurement of the study medications including batch number and expiry date will be kept in the study regulatory binder.

Study medications will be stored at the study clinic. Monthly inventories of storage conditions and stocks (medications used and remaining) will be kept at the study clinic.

Any unused primaquine after the study will be destroyed according to a protocol agreed with the Government Pharmaceutical Organisation, Thailand.

3.3.4 Packaging and Labelling of the medical products

Packaging of Primaquine Phosphate tablets: Light-resistant plastic sealable container.

Labelling: this is shown in figure 3 and Appendix 12.

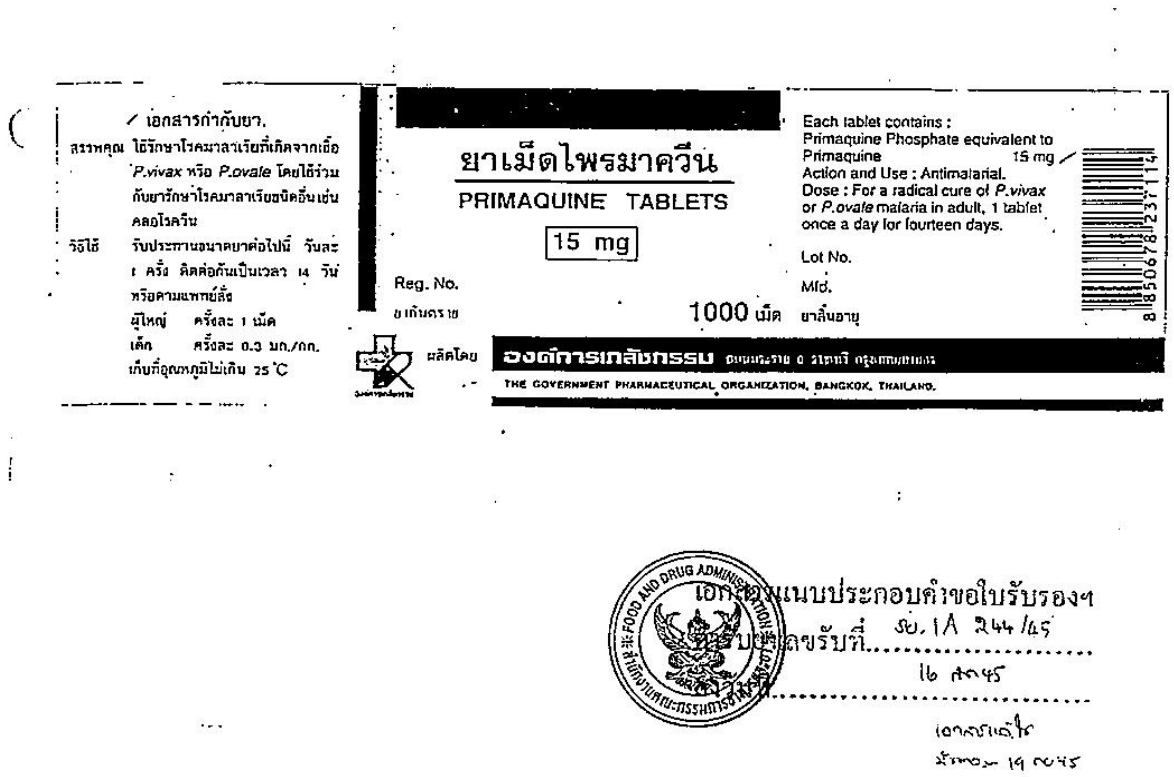

Figure 1 Medication labeling

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3.4 Estimates of quantities of each medication (presentation) to be used for the trial, and for which an import permit is needed.

Table 1 Study drug quantities

Medication name	Presentation	Quantity required
Primaquine Phosphate	Tablets (250 per bottle)	1500 tablets (6 bottles)

Section 4 Sites & Investigators

4.1 National Principal Investigator or co-ordinator (Responsible person)

Table 2 Responsible person

Name:	Chi Eziefula
Qualifications	MBBS, MRCP, MRCPath
Contact Details	+256784448758
Physical address	Walukuba Health Centre IV, Jinja, Uganda
Declaration of Capacity & Interests	[Appendix 10]

4.2 For each Site list the following:

4.2.1 Site Identifier

Name: Walukuba Health Centre IV, Walukuba, Jinja, Uganda
Postal Address: Walukuba Health Centre IV, P.O. Box 720 Jinja
Physical Address (GPS coordinates): N0 26.294' E33 13.504' 3805ft
Telephone: +256772517468
E-mail address: In charge is Dr Jenipher Namuganza jeniphernamuganza@yahoo.co.uk

4.2.2 Description of the site facilities & Staff

- Clinic and counselling rooms

The clinic room is housed in a former hospital ward. This large room is for the conduct of screening, clinical activities (review and drug administration) and pharmacokinetic study sampling

- Emergency facilities

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These are available at the Health Facility Outpatient department and there is a hospital vehicle for transfer to the Jinja Regional Paediatric Referral Hospital, which is 15 minutes drive from the Health Centre.

- Facilities for special examinations (if required)

Special examinations are not required (routine only)

- Capacity to collect, prepare, store and transport clinical samples

Capacity is available in the hospital laboratory for sample collection, preparation and storage. Samples will be transported at regular intervals by Infectious Diseases Research Collaboration (IDRC) vehicles with trained drivers.

- Storage and handling facilities for medicines

Medicines will be stored as per manufacturer's guidelines and they will be handled according to study SOPs. The study drug primaquine is to be stored in locked cupboards in a ventilated room designated as the study pharmacy. There is a window hatch to the study ward/ clinical area and to the study administrative area.

- Name and qualifications of person with responsibility for dispensing medicines

The study pharmacist is to be hired. Upon hiring, their name and qualifications will be available.

4.3 Site Principal Investigator

Name: Chi Eziefula

Qualifications: MBBS, MRCP, MRCPath

Contact Details: +256784448758

Physical address: Walukuba Health Centre IV, Walukuba, Jinja. Offices: IDRC, Mulago Hospital Complex, Kampala, Uganda

Declaration of Capacity & Interests: Please see Appendix 9

4.4 Site Sub-investigators and trial-specific support staff

SITE SUB-INVESTIGATORS:

Sarah Staedke, MD, PhD

Role in project: Co-investigator; PhD supervisor

Clinical Senior Lecturer, London School of Hygiene and Tropical Medicine, London, UK

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Co-director, Uganda Malaria Surveillance Project, Kampala,
Uganda Email: sarah.staedke@lshtm.ac.uk

Moses Kamya, MBChB, MPH, PhD
Role in project: Co-Investigator
Professor, Department of Medicine, Makerere University, Kampala, Uganda
Director, Infectious Disease Research Collaboration / Uganda Malaria Surveillance Project, Kampala
Email: mkamya@infocom.co.ug

Christopher Drakeley, PhD
Role in project: Co-investigator, PhD Supervisor/ advisor
Senior lecturer, London School of Hygiene and Tropical Medicine, London,
UK E-mail: chris.drakeley@lshtm.ac.uk

Shunmay Yeung, MRCPCH, DTM&H, PhD
Role in Project: Co-investigator, PhD Supervisor/ advisor
Clinical Senior Lecturer, London School of Hygiene and Tropical Medicine, London, UK
E-mail: shunmay.yeung@lshtm.ac.uk

Nick White, OBE, DSc, MD, FRCP, F Med Sci
Role in Project: Co-investigator, PhD Supervisor/ Advisor
Wellcome Trust Principal Research Fellow, Chairman of the Wellcome Trust South-east Asian Tropical Medicine Research Programmes, Professor of Tropical Medicine Mahidol University & Oxford University
E-mail: nickw@tropmedres.ac

Teun Bousema, PhD
Role in Project: Co-investigator; PhD advisor
Lecturer, London School of Hygiene and Tropical Medicine, London, UK
E-mail: Teun.Bousema@lshtm.ac.uk

Arthur Mpimbaza, MBChB, MMed
Role in project: Co-investigator/ collaborator
Pediatrician, Uganda Malaria Surveillance Project, Infectious Diseases Research Collaboration,
Kampala, Uganda
Email: arthurwakg@yahoo.com

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Nsobya Sam Lubwama BLT, MSc, PhD
Role in project: Co-investigator/ collaborator, laboratory director
Laboratory director, Uganda Malaria Surveillance Project, Infectious Diseases Research Collaboration, Kampala, Uganda
Email: samnsobya@yahoo.co.uk

Humphrey Wanzira, MBChB, Msc
Role in project: Co-investigator/ collaborator
Epidemiologist, Uganda Malaria Surveillance Project, Infectious Diseases Research Collaboration Kampala, Uganda
Email: wanzirah@yahoo.com

Emily Webb, PhD
Role in project: Co-investigator; statistician
Lecturer in Epidemiology and Medical Statistics, London School of Hygiene and Tropical Medicine, London, UK
E-mail: Emily.webb@lshtm.ac.uk

Please see Appendix 9 for Investigators' Declaration of Capacity & Interests

4.5 **For Hospital or Public Health Clinic Sites**

- Responsible Administrator:

Dr. Jenipher Namuganza

- Contact Details:

Telephone: +256772517468
E-mail: jeniphernamuganza@yahoo.co.uk

- Append Signed Letter of Agreement for Trial to take place. Please see Appendix 14

4.6 Append Signed Agreement/s between the Investigators and the Sponsor/s and/or Clinical Research Organization.

Please see Appendix 13.

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Section 5 Participants

5.1 Numbers of Participants as stipulated in the table below

5.1.1	Total number to be enrolled, worldwide	500
5.1.2	Total number to be enrolled in Uganda	500
5.1.3	Number of trial sites in Uganda	1
5.1.4	Intended numbers of participants at each site - evidence of availability.	500

5.2 Duration

5.2.1 Estimated trial duration: First enrolment to Final Report

1st August 2011 to 30th April 2012

5.2.2 Duration for individual Participant

- Screening period: 1 day
- Intervention period: 3 days
- Follow-up period: 28 days including enrolment day

5.3 What is the intended compensation for time and other inconvenience per participant? This should not be confused with compensation in terms of damage.
5000 UGX per day.

Section 6 History of Previous and in-progress trials

6.1 List the titles of previous trials with this (or similar) medicines in Uganda No previous trials with primaquine in Uganda

6.2 List the titles of previous trials with this (or similar) medicines in other countries

AFRICA:

Tanzania:

Bousema, T., et al., *Revisiting the circulation time of Plasmodium falciparum gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs.* Malar J, 2010. 9: p. 136.

Shekalaghe, S., et al., *Primaquine clears submicroscopic Plasmodium falciparum gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate.* PLoS One, 2007. 2(10): p. e1023.

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Shekalaghe, S.A., et al., *In Tanzania, hemolysis after a single dose of primaquine coadministered with an artemisinin is not restricted to glucose-6-phosphate dehydrogenase -deficient (G6PD A-) individuals.* Antimicrob Agents Chemother, 2010. 54(5): p. 1762-8.

Kenya:

Schneider, P., et al., *Submicroscopic Plasmodium falciparum gametocyte densities frequently result in mosquito infection.* Am J Trop Med Hyg, 2007. **76**(3): p. 470-4.

Sudan:

El-Sayed, B., et al., *A randomized open-label trial of artesunate- sulfadoxine-pyrimethamine with or without primaquine for elimination of sub-microscopic P. falciparum parasitaemia and gametocyte carriage in eastern Sudan.* PLoS One, 2007. 2(12): p. e1311.

Burkina Faso

Coulibaly, B., et al., Strong Gametocytocidal Effect of Methylene Blue-Based Combination Therapy against Falciparum Malaria: A Randomised Controlled Trial. PlosOne, 2009. 4 (5): e5318

ASIA:

Thailand:

Pukrittayakamee, S., et al., *Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria.* Antimicrob Agents Chemother, 2004. 48(4): p. 1329-34.

Cambodia:

Song, J., et al., *Rapid and effective malaria control in Cambodia through mass administration of artemisinin-piperaquine.* Malar J, 2010. **9**: p. 57.

Myanmar:

Smithuis, F., et al., *Effectiveness of five artemisinin combination regimens with or without primaquine in uncomplicated falciparum malaria: an open-label randomised trial.* Lancet Infect Dis, 2010.

SOUTH AMERICA:

Colombia:

Alvarez, G., et al., *Dynamics of Plasmodium falciparum parasitemia regarding combined treatment regimens for acute uncomplicated malaria, Antioquia, Colombia.* Am J Trop Med Hyg, 2010. 83(1): p. 90-6.

6.3 Append Interim or Final report-summaries of these trials to this application. (This may be in the Investigators Brochure or APPENDIX 11)

Please see appendix 11.

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6.4 Include a letter or certificate from the regulatory authorities in countries where previous trials have been undertaken (including those in-progress) that these trials have been GCP compliant.

Not available.

Section 7 Ethics review

7.1 Provide the local IRC approval of the Protocol for each site [Appendix 11] The protocol and the informed consent documents have been submitted for review and approval by all institutional review boards (IRBs) before the study begins. Any amendments or modifications to this material will also be reviewed and approved by the IRBs prior to implementations. The IRBs include Makerere University School of Medicine Research and Ethics Committee (SOMREC), Uganda National Council of Science and Technology (UNCST) and the London School of Hygiene & Tropical Medicine (LSHTM) Ethics Committee.

7.2 What GCP Guidelines have been followed in compiling this protocol?

GCP guidelines have been adhered to as follows:

The clinical trial will be carried out in accordance with a written protocol agreed upon and signed by the investigator and the sponsor. Any change(s) subsequently required will be similarly agreed on and signed by the investigator and sponsor and appended to the protocol as amendments.

The protocol, appendices and other relevant documentation states the aim of the trial and the procedures to be used; the reasons for proposing that it should be undertaken on humans; the nature and degree of any known risks; the groups from which it is proposed that trial subjects be selected and the means for ensuring that they are adequately informed before they give their consent.

The protocol, appendices and other relevant documentation are to be reviewed from a scientific and ethical standpoint review bodies according to local laws and regulations (in this case: institutional review board, drug regulatory authority), constituted appropriately for this purpose and independent of the investigator(s) and sponsor.

7.3 Will GCP training be provided for local staff and investigators?

Yes, GCP training will be provided for local staff and investigators who are involved in the trial.

The certificate of GCP training for the Principal Investigator is available in Appendix 9.

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Section 8 Trial conduct monitoring and reports

8.1 Describe the Safety and Monitoring Plan for each site.

Since safety forms part of the primary objective and outcome measures for this study, there is a comprehensive plan for safety and monitoring. Staff procedures will be defined in training which will be conducted prior to the start of the trial (with competence testing) and refreshed once the trial is in progress. Responsible study site staff will be trained in GCP.

Study procedures are documented in study-specific SOPs so that the study co-ordinator and Principal Investigator can monitor and assess adherence. Laboratory logs will be kept to monitor the performance of laboratory assays and to enable monitoring for abnormal laboratory results at the study site. Sample labelling will be according to SOPs and laboratory protocols will enable sample flow to be tracked at the study site.

For all clinic visits both scheduled and unscheduled, a medical clinician will be available to assess patients. Assessments will be conducted and documented in an objective manner according to SOPs. In addition, for complicated clinical issues, pathways of referral to medical specialists are outlined in SOPs. At the Regional Paediatric Hospital in Jinja, a pathway of referral has been established specifically for this trial to assist with rapid and effective management of complications. Telephone access to the Principal and main Sub-Investigators will be available to staff. The inclusion and exclusion criteria have been selected to optimise patient safety. For example, children with symptoms or signs of severe illness at baseline or who have risks for severe illness, such as anaemia or hyperparasitaemia have been excluded.

8.2 Describe the system to be used to detect, record, assign causality and the actions for adverse events.

SUMMARY OF SYSTEM FOR PHARMACOVIGILANCE

Assessment for adverse events will be conducted in a systematic and objective fashion on each day of scheduled and unscheduled follow-up. Adverse events will be recorded on a separate adverse event reporting form. The severity of abnormal symptoms, signs and laboratory parameters will be graded. The causal association of adverse events with use of study medication will be graded.

IDENTIFICATION AND RECORDING OF ADVERSE EVENTS

Participants will be monitored for adverse events on each day of scheduled follow up and on unscheduled follow up visits. This will involve the identification of any new signs or symptoms that were not present on the previous visit.

Adverse events will be recorded on a separate adverse event reporting form (Appendix Q). The following data will be collected on adverse events:

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-Description of adverse event
-Date of adverse event onset
-Date adverse event reported
-Maximum severity of the adverse event
-Maximum suspected relationship of the adverse event to the study medication
-Is the adverse event serious?
-Is the adverse event unexpected?
-Identification of the person reporting the adverse event -Was the event episodic or intermittent in character? -Outcome of the adverse event
-Date of resolution of the adverse event

Duration of follow up: Adverse events will be followed up until they have resolved or stabilized in the opinion of the study clinician, even in the event that this exceeds the end of the study or following a patient's withdrawal from the study.

GRADING OF SEVERITY OF ADVERSE EVENTS

The severity of adverse events (symptoms, signs, abnormal laboratory parameters) will be graded according to a system developed by the UMSP/ IDRC which are in accordance with guidance from the NIH Division of Microbiology and Infectious Diseases (DMID) toxicity tables and the WHO Toxicity grading scale for determining the severity of adverse events.

ANY clinical event deemed by the clinician to be serious or life-threatening is considered a grade 4 event. Clinical events considered to be serious or life-threatening include, but are not limited to: seizures, coma, tetany, diabetic ketoacidosis, disseminated intravascular coagulation, diffuse petechiae, paralysis, acute psychosis, severe depression. The grading of severity of adverse events is summarized in the table below.

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Grading of severity of adverse events:

Grade	Severity	Description
1	Mild	Transient or mild discomfort; no limitation in activity; no medical intervention/therapy required
2	Moderate	Mild to moderate limitation in activity – some assistance may be needed; no or minimal medical intervention required
3	Severe	Marked limitation in activity; some assistance usually required; hospitalization possible
4	Life-threatening	Extreme limitation in activity, significant assistance required; significant medical intervention/therapy required; hospitalization probable
5	Death	

The causal association of adverse events with use of study medication is summarized in Appendix S of the full study protocol.

REPORTING OF SERIOUS ADVERSE EVENTS

Periodic summaries of all adverse events will be compiled by the principle investigator and submitted to the DSMB. Reporting of serious adverse events, fatal/ life-threatening events will be according to the requirements of the IRBs (SOMREC, LSHTM, and UNCST) and the NDA.

The guidelines for the local IRBs are as follows:

Institution	Type of Adverse Events	When to Report
UNCST	• All Serious* or Unexpected± events irrespective of relationship	• Death and Life-threatening events within 48-hours by phone, fax or email with report submitted within 7-calendar days • All other reportable events within 15-calendar days of awareness
MU SOMREC	• All Serious* or Unexpected± events irrespective of relationship	• Within 7-working days of awareness
LSHTM	• All Serious* or Unexpected± events irrespective of relationship	• Prompt reporting- **All SAEs should be reported to the PI**

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		within 24hours , PI should ensure that all SAEs are in annual report • Prompt reporting to QA/QC manager, RA, REC- **Any SAE that is serious, suspected of having relationship to Trial drug and is unexpected (SUSAR)**

Serious Adverse Event (SAE) is any AE that results in any of the following outcomes:

- Death,
- Life-threatening adverse experience
- Inpatient hospitalization or prolongation of existing hospitalization,
- Persistent or significant disability/incapacity,
- Congenital anomaly/birth defect, or cancer, or
- Any other experience that suggests a significant hazard, contraindication, side effect or precaution that **may require medical or surgical intervention** to prevent one of the outcomes listed above,
- Event occurring in a gene therapy study
- Event that changes the risk/benefit ratio of the study.

±***Unexpected Adverse Event.*** An adverse event is defined as being unexpected if the event exceeds the nature, severity, or frequency described in the protocol, consent form and investigator brochure (when applicable). An unexpected AE also includes any AE that meets any of the following criteria:

- Results in subject withdrawal from study participation,
- Due to an overdose of study medication, or
- Due to a deviation from the study protocol

8.3 Describe the actions to be taken following reports of Serious Adverse Events.

DAIDS Grade 1 or 2 Toxicities

Participants experiencing grade 1 or 2 toxicities and/or adverse events will be managed at the discretion of the site investigator and healthcare worker.

DAIDS Grade 3 Toxicities

Participants experiencing grade 3 toxicities will be referred to a clinician for immediate evaluation. If the study drug primaquine/ placebo has not yet been given, it may be withheld at the site investigator's discretion. Clinicians will be encouraged to consult

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with specialists at the Jinja Paediatric Regional Referral Hospital. Participants should be re-evaluated every 2 -3 days if possible (if the patient is able to return for follow-up on that schedule), until the adverse event returns to ≤ grade 2 or until stabilized and no longer in need of frequent monitoring, to be determined by the site investigator.

DAIDS Grade 4 Toxicities

If a grade 4 adverse event or toxicity develops, the study drug should be withheld at the discretion of the site investigator if it has not yet been given. Appropriate consultations should be made to specialists at the Jinja Paediatric Regional Referral Hospital or Mulago National Referral Hospital and further consultations/ referrals made at the discretion of the site investigator. The patient should be monitored frequently until the adverse event returns to ≤ grade 2 or until stabilized and no longer in need of frequent monitoring, to be determined by the site investigator.

8.4 Describe the composition and remit of the Data Safety Monitoring Board or similar body. Include conditions for Pause- or Stop- rules.

A data and safety monitoring board comprising clinicians (including local specialist), a statistician and including clinical trials expertise, will review the study protocol prior to implementation of the trial and will be convened to review the study periodically. The agenda for each meeting will be made in conjunction with the Clinical Trials Unit (CTU) at LSHTM and the DSMB Chair. The CTU is responsible for quality assurance in clinical trials sponsored by LSHTM.

All study data and interim results will be presented to the DSMB using treatment group codes (A, B, C, or D) that will correspond with, but not identify, the actual treatment groups. Master copies of the randomization code and treatment group assignments will be held in the administrative offices in Kampala and London.

Guidelines for stopping the study due to safety outcomes will be developed and established by the DSMB.

8.5 When will Interim Reports be submitted?

Information reflecting study progress and data quality and safety and tolerability data will be provided to the DSMB at regular intervals for review in accordance with the schedule they recommend. The timing of interim analysis is expected to be after 250 patients are enrolled and will be confirmed with the DSMB.

The Interim report to the review bodies will summarise any adverse events.

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8.6 Final Report - Estimated due-date?

The final report summarizing adverse events is expected within 15 calendar days after the last patient has completed 28 days of follow up. This is expected by 30th April 2012.

Section 9 Insurance

9.1 Provide a copy of the current insurance certificate. (APPENDIX 9)

Please see Appendix 9.

9.2 Provide evidence that each member of the Investigator team is covered by relevant Malpractice insurance for this trial

Please see Appendix 9 for details of the malpractice coverage for study personnel.

Section 10 Description of the Trial

10.1 Is the Title of the Trial fully descriptive?

The trial is a double-blinded randomized, placebo-controlled clinical trial with four parallel arms to evaluate the efficacy and safety of variable dose primaquine for clearance of gametocytes in uncomplicated falciparum malaria in children in Uganda.

10.2 Summarized Rationale for this Clinical Trial, including relevance to Uganda

Malaria is a major public health problem. Every year, approximately one million people die from malaria and the majority of these are children aged less than five years. Globally, most deaths are due to *P. falciparum* malaria. This is the most prevalent species of malaria in Africa. The five countries with the greatest number of malaria deaths in the world are Uganda, DRC, Nigeria, Ethiopia and Tanzania. Current malaria control efforts are inadequate, despite a new drive for malaria elimination since 2007. Therefore, it is important that new tools are evaluated for use in malaria control.

Malaria is transmitted from mosquitoes to humans through the bite of the mosquito. The mosquito injects malaria parasites from its mouthparts into the human bloodstream. In the human, the malaria parasite changes into sexual forms called gametocytes, and it is these that are infectious to mosquitoes. Onward transmission back to the mosquito occurs when it feeds on an infected human.

Gametocytocidal drugs (drugs which destroy gametocytes) are now assuming a high profile as a tool for blocking transmission of falciparum malaria. Artemisinin derivatives have some gametocytocidal action, being effective against developing gametocytes (stages 1 to 3 gametocytes). This may explain the reduction in malaria transmission in

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settings where their use is well-established. However, following artemisinin combination therapy, microscopic and sub-microscopic (measured using molecular techniques) gametocytaemia is still detectable and individuals are still infectious to mosquitoes, i.e. transmission to mosquitoes can still occur. The only drugs available which are highly effective against mature gametocytes (stages 4 to 5) are the 8-aminoquinolines. Drugs in this class include primaquine and newer compounds such as tafenoquine and bulaquine. Of these, primaquine is the least expensive (cost of 69 Ugandan shillings per dose of 15mg) and most widely available.

The WHO recommends that, to block transmission of falciparum malaria, a single dose of primaquine should be added to standard treatment regimes (artemisinin-containing therapy, or "ACT") in malaria control and eradication programmes (WHO, Malaria Treatment Guidelines 2010). Primaquine acts against the gametocytes of the falciparum malaria parasite (the form of the parasite which is responsible for onward transmission from humans to mosquitoes). Primaquine is the only widely-available and affordable drug with this action, so it is likely to have an important role in blocking the transmission of malaria.

The dose of primaquine recommended by the WHO is 0.75mg/kg. However the dose of primaquine for optimal safety and efficacy has never been evaluated in clinical trials. This is important because primaquine has a dose-dependent risk of causing haemolysis (destruction of red blood cells) in pre-disposed individuals, such as those with G6PD deficiency. The higher the dose, the higher the risk of haemolysis. G6PD deficiency is a condition which is prevalent in malaria-endemic areas such as Uganda. Therefore, it is essential that data on primaquine's safety is available in such areas before the WHO recommendations are put into practice. Lower doses should have less impact on haemoglobin than the WHO-recommended dose of 0.75mg/kg.

Following the WHO recommendations and renewed calls for malaria elimination, primaquine is rapidly generating interest and in several countries, already it is being used as a transmission-blocker. It is estimated that millions of people stand to receive doses for this purpose annually. As malaria control and elimination programmes are developed across Africa, primaquine is likely to assume an important role.

Few studies have looked at the effect of lower doses of primaquine than the dose recommended by the WHO for transmission-blocking. Those that have looked have found that lower doses are still able to reduce transmission/ gametocytes when compared to placebo (non- active drug). No studies have compared the WHO dose to lower doses. None have been powered (large enough) to assess safety outcomes and no studies have documented the pharmacokinetics of primaquine in African children. Pharmacokinetic data provides an understanding of how the drug is handled in the body in specific

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populations and age groups. This data is important when developing appropriate drug dosing strategies.

We hypothesise that lower doses of primaquine have a significantly lower risk of, or an absence of adverse effects compared to the WHO-recommended dose, but retain the transmission-blocking effect.

We propose to test this hypothesis in a four-arm placebo-controlled clinical trial with a non-inferiority design to evaluate the safety and efficacy of the WHO dose (0.75mg/kg) and lower doses of primaquine in combination with ACT for clearance of *P. falciparum* gametocytes in children in Uganda. The study will include a pharmacokinetic analysis.

10.3 BRIEF Background information should include:

- **The disease or condition and local epidemiology**
- **Properties of the medicine - hypothesis for action**
- **Description of risks of the protocol and the potential harms of the medicine.**
- **Pre-clinical animal toxicology test results in-animals and in-vitro that establishes probable safety and efficacy in humans**
- **Prior Clinical trial report summaries that establishes probable safety and efficacy in humans**
- **Include evidence that the formulations used in the pre-clinical and previous studies are identical to that in this application. Any variations should be highlighted and justified.**
- **Published reviews or reports relevant to this disease and this type of medicine**

- **The disease or condition and local epidemiology**

The plasmodial parasite, malaria, infects an estimated 450 million people globally each year. The majority of these infections occur in Sub-Saharan Africa where the predominate species, *Plasmodium falciparum*, is responsible for the greatest proportion of deaths worldwide due to malaria. Aside from directly-attributable morbidity and mortality from severe malaria, malaria is responsible for a substantial all-cause mortality and morbidity which is contributed to by anaemia, adverse pregnancy outcomes for mother and child and long term sequelae of infection.

The burden of malaria in Uganda is high. Uganda is one of 5 countries with the highest global incidence of deaths and morbidity from malaria.

In Uganda, malaria transmission intensity is highly heterogenous. In the south western districts such as Kabale and Kanungu, transmission is low and in mountainous areas, there is little or no malaria leaving the areas prone to epidemics. In southern urban areas, malaria endemicity is characteristically medium to high. In the East and Northern regions of the country malaria transmission is high or very high. In Apac very high transmission intensity has been recorded of 1500 infective bites per person per year. This

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heterogeneity calls for a range of interventions to control and ultimately eliminate malaria from Uganda.

- **Properties of the medicine - hypothesis for action**

Primaquine is an old drug, developed in the 1940s and in widespread use since the 1960s. It was one of the first synthetic antimalarials to be developed. It belongs to the 8-aminoquinoline drug class. Other drugs in this class include Tafenoquine and Bulaquine, but these are not yet widely available. The 8 -aminoquinolines are gametocytocidal, that is, they are active against the sexual forms of the *P. falciparum* malaria parasite, the gametocytes. These blood-borne sexual stages, although harmless to humans, are infectious to mosquitoes and are responsible for onward transmission of malaria from human to mosquito.

Primaquine is also effective against the sporozoites of *Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium falciparum* leading to its use as a prophylactic. It has no effect on the blood stages of *Plasmodium falciparum.* Primaquine is most widely used for its effect against *P. vivax* and *P. ovale* hypnozoites as anti-relapse therapy (PART). For this purpose, it has been used for decades. In adults, the dosing of primaquine for PART is 30mg daily for two weeks. This is a total dose of 420mg. In contrast, the WHO-recommended dose for *P. Falciparum* transmission-blocking is one single dose of 0.75mg/kg, a substantially smaller dose.

- **Description of risks of the protocol and the potential harms of the medicine.**

Risks of the study drug, primaquine, include abdominal symptoms (nausea, vomiting, abdominal discomfort) which are reduced by administration with food, methaemoglobinaemia (mild cyanosis may occur) and haemolysis (fall in blood count). In previous studies in East African children using the WHO-recommended dose of primaquine, (no higher doses will be given in the study) no subject had a fall in blood count that caused symptomatic anaemia, or required a blood transfusion or hospitalization. No serious adverse events have been recorded in other large studies using the WHO-recommended single dose of primaquine, 0.75mg/kg. Haemolysis is an outcome to which study clinicians will be highly alert and respond with prompt and appropriate SOP-guided management. As a precaution, in the case that specialist care or blood transfusion is required, referral systems to Jinja Paediatric Referral Hospital and procurement of blood have been optimised.

To avoid administration in pregnancy, females will be asked if they have started menstruating. If they have, or they give a history that they are pregnant or breastfeeding, they will not be enrolled in the study.

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The research will not have a direct benefit to the individual subject, but by clearing gametocytes and preventing the individual from transmitting malaria on to mosquitoes, primaquine has potential for reduction of transmission at the community level. The aim of this study is to provide information in the safety and dosing of primaquine for use in African malaria elimination and control programmes.

Other protocol risks to participants include the risks of blood sampling procedures include pain, transient bleeding and soft-tissue infection. These will be minimized by adhering to strict protocols for cleaning skin and taking samples.

- **Pre-clinical animal toxicology test results in-animals and in-vitro that establishes probable safety and efficacy in humans**

These can be found in section 12 of the Investigator's Brochure.

- **Prior Clinical trial report summaries that establishes probable safety and efficacy in humans**

These can be found in section 13 of the Investigator's Brochure.

- **Include evidence that the formulations used in the pre-clinical and previous studies are identical to that in this application. Any variations should be highlighted and justified**

All studies used generic Primaquine Phosphate with the identical active ingredient. The following studies used exactly the same formulation as that in this application:

Smithuis, F., et al., *Effectiveness of five artemisinin combination regimens with or without primaquine in uncomplicated falciparum malaria: an open-label randomised trial.* Lancet Infect Dis, 2010.

Pukrittayakamee, S., et al., *Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria.* Antimicrob Agents Chemother, 2004. 48(4): p. 1329-34.

- **Published reviews or reports relevant to this disease and this type of medicine**

These can be found in Appendix 11.

For full background information, please see pages 14-22 of the full trial protocol (Appendix 1).

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10.4 Objectives of this trial

Justification for objectives is given in italics.

GENERAL OBJECTIVE

To evaluate the efficacy and safety of different doses of primaquine administered with ACT for the purpose of reducing *P. falciparum* gametocytes in the infected human to prevent transmission of falciparum malaria to the anopheles mosquito.

SPECIFIC OBJECTIVES

1. To evaluate the efficacy of different doses of primaquine when administered with AL (artemether-lumefantrine, an ACT) as measured by gametocyte prevalence and density

Gametocyte prevalence and density are a measure of the transmission potential of the human host to mosquitoes.

2. To evaluate the safety of different doses of primaquine when administered with AL as measured by change in mean haemoglobin, prevalence of severe anaemia (Hb <5g/dL), and evidence of black urine (haemoglobinuria; dipstick positive)

Haemoglobin level, anaemia and black urine (secondary to haemolysis) are all the adverse side effects of primaquine that this study seeks to evaluate. Although severe haemolysis is not expected with single dose primaquine, it is essential that this is captured in an objective.

3. To assess the safety of different doses of primaquine when administered with AL as measured by prevalence/ incidence of adverse events and tolerability

This forms the essential pharmacovigilance for this trial.

4. To assess factors impacting the efficacy and safety of different doses of primaquine when administered with AL such as age, gender, pre-treatment level of gametocytes, G6PD enzyme function and G6PD genotype

This is incorporated to provide data on confounding factors for primaquine's efficacy and safety. This is to help inform operational programmes on the generalisability of this study, given this specific population and transmission setting.

5. To obtain basic pharmacokinetic parameters for primaquine in the study population

Pharmacokinetic data provides an understanding of how the drug is handled in the body in specific populations and age groups. This data will help define a dosing strategy for primaquine.

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10.5 Trial Design: Describe and justify each component.

10.5.1 Phase:
This is a phase III trial according to the NDA definition: "with the purpose of determining the short- and long-term safety/efficacy balance of formulation(s) of the active ingredient, and of assessing its overall and relative therapeutic value."

Placebo or comparator:
The two test doses of primaquine are compared to placebo and to the comparator (WHO recommended) dose of primaquine 0.75mg/kg. Hence the study has four arms.

Randomization and blinding:
After enrollment, participants will be assigned to a treatment group using a randomized method stratified by sex. The responsible study staff will select sequential opaque envelopes (from either the male or female pile). Each envelope contains a pre-determined treatment assignment code. The study nurse will bring the envelope to the study pharmacist.

The study pharmacist will possess the assignment code breaker and will dispense the relevant treatment for days 0-2. The treatment assignment code corresponds to a PQ dose to be given on day 2: P0 (placebo), P1-3 (variable dose primaquine) and the study pharmacist has access to the code but the study nurses and clinicians do not. Having selected an opaque envelope for the child, the study nurse will bring the envelope to the study pharmacist. The study pharmacist will open the envelope, document the treatment assignment code and the participant's study number on the treatment assignment log, calculate the correct dose of primaquine/ placebo in milligrams and document the number of millilitres of primaquine/ placebo solution that are required. The treatment assignment code and the dose to be given will not be documented on the CRF or provided to the study nurse.

The study pharmacist will be the only member of the clinic team not blinded to the treatment groups. The study pharmacist will not have patient contact and will not be involved in assessing patients or assigning outcomes.

The study site staff who are administering drugs assessing patients and processing laboratory samples will not have access to the randomization code breaker.

The participant will not be informed of the PQ dose to be administered

The primaquine dose will be placebo-controlled. All participants will receive a second treatment on day 2. Placebo will be as indistinguishable as possible from PQ, both being dissolved tablets in solution.

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10.5.2 Time sequence –
A Table of screening, intervention and follow-up visits will be of assistance.

Day of follow up	0	1	2	3	7	10	14	21	28	Unscheduled
CLINICAL:										
Recruitment	X									
Screening interview	X									
Informed consent	X									
Clinical screening (history and examination)	X									
Randomization	X									
History	X	X	X	X	X	X	X	X	X	X
Tympanic temperature	X	X	X	X	X	X	X	X	X	X
Physical examination	X	X	X	X	X	X	X	X	X	X
Assessment for adverse events	X	X	X	X	X	X	X	X	X	X
Complete case record form	X	X	X	X	X	X	X	X	X	X
TREATMENT:										
ACT	X (1^{st})	X (2^{nd})	X (3^{rd})							
Primaquine (PQ)			X							
LAB TESTING:										
Finger prick sample	**Sample collected into EDTA eppendorf then pippetted in the lab**									
Blood smear	X	X	X	X	X	X	X	X	X	X
Filter paper W#3 + W#903	X	X	X	X	X	X	X	X	X	X
L6 buffer	X	X	X	X	X	X	X	X	X	
Haemoglobin (Hemocue®)	X	X	X	X	X	X	X	X	X	
Phlebotomy sample										
G6PD (serum)							X			

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10.5.3 Participants

Eligibility

Complete selection criteria are listed as follows:

Inclusion criteria	**Justification**
1. Age >/ 1 year and </10 years	Limited to define population. Highest prevalence of clinical malaria in this age group in this region.
2. Weight over 10kg	For safety: pharmacokinetics not defined in infants
3. Fever >38 degrees C (tympanic) or history of fever in the last 24 hours	Clinical malaria
4. *P. falciparum* parasitaemia <500 000/µl	Excluding hyperparasitaemia which could be a risk factor for the development of severe malaria prior to administration of the study drug
Exclusion criteria	**Justification**
1. Enrolled in another study	To avoid complication for participants and risks of dual interventions
2. Evidence of severe illness/ danger signs	To maximize safety and avoid confounders
3. Known allergy to study medications	To maximize safety
4. Haemoglobin< 8g/dL)	To maximize safety
5. Started menstruation	To avoid administration of primaquine in pregnancy
6. Pregnancy (by history) or breastfeeding	Primaquine is contra-indicated in pregnancy or breastfeeding
7. Primaquine taken within the last 4 weeks	To avoid confounding of the efficacy effect and avoid double dosing
8. Blood transfusion within the last 90 days	To ensure that G6PD results are reliable
9. Non-falciparum malaria co-infection	To define the study population

10.5.4 Treatment regimens for each group.

The table in 10.5.2 above can be used to set this out

All enrolled individuals will receive a full three-day course of AL, and will be randomized to receive a dose of primaquine or placebo with their last dose of AL on day 2.

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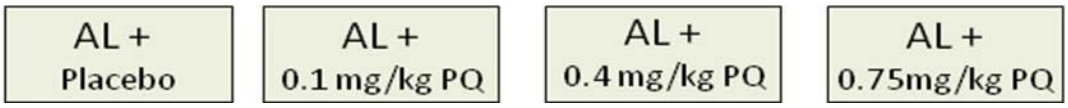
There are four treatment arms as follows:



10.5.5 Follow-up, sampling collection and monitoring plans; Immediate monitoring - intermediate monitoring - long term monitoring Diary cards

Enrolled participants will receive ACT treatment for their malaria infection on days 0, 1 and 2. On day 2, they will receive a dose of primaquine or placebo (one of four treatment arms). All study medications will be given under direct observation and participants will be monitored for a minimum of 30 minutes after the last dose of AL to assess for vomiting (which is managed according to an SOP).

Participants will return for follow up on days 3, 7, 10, 14, 21, and 28. On each day of follow up, there will be an assessment by a clinician and an assessment for adverse events and blood samples taken. If participants desire to be seen on other days, they are encouraged to come to the study clinic for any medical concerns or simply to contact the study team to ask questions. The blood samples will be taken by finger prick unless this method fails (unexpected), in which case, blood will be taken from a vein. The blood will be dropped onto a glass slide to make malaria blood films to examine the number of parasites and species, dropped onto a haemoglobin meter to assess the blood count and dropped onto filter paper for the following tests:

- Gametocyte detection. This will be tested in London/ Nijmegen because facilities are not available in Uganda
- G6PD enzyme function. This will be tested in Kampala.
- G6PD genotype. This will be tested in Uganda as long as facilities remain available and also in London (confirmatory tests), where facilities are guaranteed.

Remaining blood spots will be stored in London for future research, with subjects' consent.

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As per section 10.5.2, the sample collection schedule is as follows:

Day of follow up	0	1	2	3	7	10	14	21	28	Unscheduled
Finger prick sample	**Sample collected into EDTA eppendorf then pippetted in the lab**									
Blood smear	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Filter paper W#3 + W#903	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
L6 buffer	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Haemoglobin (Hemocue®)	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	
Phlebotomy sample										
G6PD (serum)							**X**			

Additional diary cards will not be used. Primaquine is a relatively short-acting drug. The half life is 1-6 hours and after 24 hours, the parent drug is rarely detectable in the blood. The timescale for effect of the study drug is expected to be detected within the monitoring framework of the study.

Telephone access to investigators:

Telephone numbers (mobile phones) of the principal investigator and two alternative responsible clinicians will provided to all participants on the patient information leaflet and participants/ parents/ guardians will be encouraged to use the telephone number if required.

10.6 Outcomes Measurements and Analysis

10.6.1 Describe each outcome/variable (including safety) and explain or justify

	OUTCOME MEASURE	DESCRIPTION
EFFICACY		
PRIMARY	**Mean number of days to gametocyte clearance (gametocyte clearance time, GCT)**	**Mean number of days per treatment arm for gametocytes to become undetectable using sub-microscopic molecular testing methods (QT-NASBA). -Re-appearance of gametocytes after day 14 will be considered re-infection and excluded.**

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	SECONDARY	Mean (+/ - SD) area under the curve of gametocyte density per day during 14 days of follow-up	Total number of gametocytes (measured by QT-NASBA) seen over follow up, averaged per day of follow up (days 0-14)
		Point prevalence of gametocytes on days 7, 10 and 14	Mean number of gametocytes (measured by QT-NASBA) per treatment arm on days 7, 10 and 14
		Proportion (%) of participants with gametocytes on each day of follow up	For each treatment arm, percentage of participants with gametocytes (measured by QT -NASBA) on each day of follow up from days 0-14.
SAFETY			
	PRIMARY	**Mean (+/- SD) maximal fall (+/ or -) in Hb (g/dL) from enrollment to day 28 of follow-up**	**Mean maximal greatest negative difference in Hb (measured by Hemocue®) from enrollment value per treatment arm over 28 days follow up**
	SECONDARY	Follow-up day of Hb nadir	Mean day of follow up (day 0-28) per treatment arm of lowest Hb measurement (by Hemocue®)
		Maximal percentage fall in Hb level compared to enrolment value	Size of maximal Hb drop (by Hemocue ®) during follow up (day 0-28) from enrollment value, divided by enrollment value, *100
		% participants with Hb < 5g/Dl during follow up	Percentage(number) per treatment arm during days 0-28
		Requirement for blood transfusion	Percentage (number) of children receiving blood transfusion per treatment arm during days 0-28
		Evidence of black urine	Percentage (number) of children with documented black/ dark urine with urine dipstick positive for Hb per

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		treatment arm during days 0-28
	Incidence of serious adverse events by sign, symptom, laboratory parameter and relationship to taking study drug	Percentage (number) per treatment arm during days 0-28
	Incidence of gastrointestinal symptoms after taking study drug	Percentage (number) per treatment arm during days 2-7

10.6.2 Describe the samples that will be collected and the analyses to be conducted on each sample

This is described in section 10.5.5

10.6.3 Provide evidence that the Laboratories that will conduct the Safety screening, and the End-point assays are accredited and competent to do the assays. (APPENDIX 7)

Laboratory analysis will be performed at the central IDRC laboratory in Kampala. This is a research laboratory which does not have accreditation, but all operations are according to approved SOPs and protocols. Quality assurance is adhered to with internal and, where appropriate, external validation of assays. Staff are trained in Good Laboratory Practice. This also applies to the research laboratories at London School of Hygiene and Tropical Medicine, the Radboud University of Nijmegen in the Netherlands, where the QT-NASBA analysis will be conducted and the Mahidol University Pharmacology laboratory in Thailand. The following assays will be performed at the study site: Hemocue®, initial malaria slide reading, G6PD qualitative analysis. These will be according to protocols and SOPs. Internal quality control will be conducted with each Hemocue and G6PD assay. The IDRC laboratory will provide quality control for malaria slide readings.

10.6.4 Describe the intended statistical analysis to be conducted. Provide evidence that the study is powered to provide the intended outcome.

Intended statistical analysis

Please refer to section 5.2 of the full study protocol for a description of the planned statistical analysis.

It is notable that analyses will be undertaken as “intention-to-treat” (including all individuals randomized). In addition, since ITT analysis may increase the risk of falsely

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claiming non-inferiority, a "per-protocol" analysis (including all individuals followed up as per protocol) will also be undertaken.

Sample size calculations
For efficacy, the sample size calculation is based on non-inferiority of each of the two test dose arms to the comparator arm, the WHO-recommended dose of PQ, 0.75mg/kg. The non-inferiority margin for days to gametocyte clearance is proposed as 2.5 days, taking into consideration data from previous studies. The addition of primaquine to ACT in Tanzania reduced the time to gametocyte clearance from 28.6 to 6.3 days. We used the size of this difference to consider a clinically-acceptable inferiority margin. Allowing for 10% loss to follow up, a sample size of 120 per arm will provide over 80% power at the 0.05 significance level to detect non-inferiority to the standard arm. This sample size also allows for an analysis of superiority of the efficacy of the two test dose arms to placebo.

For safety, the sample size calculation is based on superiority of each of the two test dose arms to the comparator arm, the WHO-recommended dose of PQ, 0.75mg/kg. It is important that adequate numbers of G6PD deficient individuals are incorporated per group in order to enable appropriately-powered subgroup analyses because this is the subgroup where the fall in Hb after ACT/PQ treatment is expected to be largest. Expecting that 16% of males will be G6PD-hemizygous (from previous survey data), and given an overall mean absolute drop amongst the G6PD-deficient individuals of 2.5g/dL with SD 2.6 (from relevant Tanzanian data), a sample size of 113 per arm would be required to detect that drop. Allowing for 10% loss to follow up, this would require a sample size of 125 per arm. Hence, a total sample size of 500 will provide adequate power to analyse both primary outcomes.

10.7 Are any Sub-studies intended? Provide full details.
A pharmacokinetic sub-study will be conducted.

Pharmacokinetic data describes how a drug is managed (and metabolized) in different groups of individuals.

Studies that provide pharmacokinetic data on primaquine have been conducted largely in Southeast Asia and Australasia. The majority of studies have been on adults. There is a lack of data on the pharmacokinetics and pharmacodynamics of primaquine in African children. Given that primaquine may be deployed in malaria endemic areas in Africa, this data is needed.

There will be a separate informed consent form for this. The details of the pharmacokinetic sub study are as follows:

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Pharmacokinetic evaluations will be obtained on approximately one quarter of the enrolled participants; a maximum of 160 participants will be recruited for pharmacokinetic sampling. There will be a separate consent process for this evaluation. Participants will be consented for this on day 1 and asked to come for sampling on days 2 to 4. The sampling on day 2 will happen whilst they are at the clinic for their last day of AL and the study dose of PQ/ placebo.

The pharmacokinetic sampling will involve taking a total of 7 venous blood samples of less than 2mls. The total amount sampled, being approximately 11-14 mls in 3 days. The first sample is just prior to the PQ/ placebo dose (a baseline sample) and the subsequent six doses are at intervals up to 72 hours after the dose of primaquine/ placebo. The blood samples will be taken at fixed times between 8am to 5pm. Participants will have to attend the clinic a minimum of 30 minutes prior to this to enable preparation for sampling. The first 5 samples are taken on day 2 and they will be taken through a venflon, sited when the baseline pharmacokinetic sample is taken. If a venflon is not sited successfully, a butterfly needle may be used. The last two samples (one on day 3 and one on day 4) will be taken by individual blood draws (venepuncture). The participant will be asked to stay in the clinic between sampling times on day 2.

In order to minimize the total number of blood draws per participant, the sampling timeframe has been randomized so that over the total population of participants, a population pharmacokinetic model can be constructed for analysis. Six randomized sample times will be allocated to sequential consenting participants in opaque envelopes. Each sample time is within a window so that there are 5 samples on day 2 and one each on days 3 and 4.

Pharmacokinetic samples will be analysed in Professor Niklas Lindegardh's laboratory in Mahidol University, Bangkok, Thailand, where the randomized sampling framework was generated.

10.8 Are any genetic studies (HLA-typing or gene marker analysis) intended? Provide full details, and justify this.

G6PD genotyping analysis will be performed. This will be by PCR for the most common G6PD alleles in East Africa. G6PD analysis forms a crucial part of the analysis, given that it is a risk factor for haemolysis with primaquine.

Is there a separate Informed Consent Form for this?

The Informed Consent for these genetic analyses is included in the consent for participation in the study and in the consent for future use of biological specimens.

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10.9 Will clinical samples be stored for any period beyond the duration of this trial?

Yes: informed consent is requested for the future use of samples by other investigators and storage of samples outside Uganda.

10.9.1 What is the purpose of such archiving?

It is intended that the samples may be available for further work on malaria research. Samples will be used only for research. They will not be sold or used for the production of commercial products.

10.9.2 What controls are to be placed on their confidentiality and possible future use?

No genetic information obtained from this research will be placed in participants' medical records. These samples will be identified only by codes so that they cannot be readily identified with the patient. Therefore, all study staff using the specimens will not be able to readily find out the name of the participant.

10.10 Participant Information Leaflet (PIL) and Informed Consent (ICON)

10.10.1 Append a copy of the PIL & ICON [Appendix 3] Please see Appendix 3.

10.10.2 In what languages will this be available?

This will be available in English, Luganda, Lusoga and Kiswahili.

10.10.3 Append the Parent / guardian consent form, in the case where minor participants will be included.

Please see Appendix 3.

10.10.4 Are there separate ICON for sub-studies or Genetic studies?

Yes, there is a separate ICON for the pharmacokinetic sub-study. Please see Appendix 3.

10.11 Publication Policy

Provide details of the Investigators and Sponsors intentions and freedom to publish the outcomes of this study.

The findings of this study may be published in a medical journal in accordance with UNCST, Makerere University, Wellcome Trust and LSHTM guidelines. They may also be presented at relevant academic conferences and meetings.

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Appendices

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APPENDIX 1: Trial Protocol

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APPENDIX 2: Investigators Brochure

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APPENDIX 3: Participant Information Leaflet and Informed Consent

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APPENDIX 4: Certificate of GMP manufacture of the trial medicine or other evidence of manufacture quality, safety and consistency

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APPENDIX 5: Package Inserts for trial medicines.

- ✓ ***Primaquine Sanofi Aventis package insert***
- ✓ ***Artemether-Lumefantrine Ajanta Pharma Ltd package insert***

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APPENDIX 6: Certificate of GMP manufacture of the placebo - if appropriate.

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APPENDIX 7: Evidence of accreditation of the designated Laboratories or other evidence of GLP and assay validation.

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APPENDIX 8: Insurance Certificate specific for the trial

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APPENDIX 9: Signed and completed Declarations by all Investigators

- ✓ ***Investigator Declarations***
- ✓ ***Investigator CVs***
- ✓ ***GCP certificate of Principal Investigator***

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APPENDIX 10: Approval of Ethics Committees for the Protocol

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APPENDIX 11: Full, legible copies of key, peer-reviewed published articles supporting the application.

Articles provided:

Bousema, T., et al., *Revisiting the circulation time of Plasmodium falciparum gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs.* Malar J, 2010. 9: p. 136.

Shekalaghe, S., et al., *Primaquine clears submicroscopic Plasmodium falciparum gametocytes that persist after treatment with sulphadoxine-pyrimethamine and artesunate.* PLoS One, 2007. 2(10): p. e1023.

Shekalaghe, S.A., et al., *In Tanzania, hemolysis after a single dose of primaquine coadministered with an artemisinin is not restricted to glucose-6-phosphate dehydrogenase -deficient (G6PD A-) individuals.* Antimicrob Agents Chemother, 2010. 54(5): p. 1762-8.

Schneider, P., et al., *Submicroscopic Plasmodium falciparum gametocyte densities frequently result in mosquito infection.* Am J Trop Med Hyg, 2007. **76**(3): p. 470-4.

El-Sayed, B., et al., *A randomized open-label trial of artesunate- sulfadoxine-pyrimethamine with or without primaquine for elimination of sub-microscopic P. falciparum parasitaemia and gametocyte carriage in eastern Sudan.* PLoS One, 2007. 2(12): p. e1311.

Coulibaly, B., et al., Strong Gametocytocidal Effect of Methylene Blue-Based Combination Therapy against Falciparum Malaria: A Randomised Controlled Trial. PlosOne, 2009. 4 (5): e5318

Pukrittayakamee, S., et al., *Activities of artesunate and primaquine against asexual- and sexual-stage parasites in falciparum malaria.* Antimicrob Agents Chemother, 2004. 48(4): p. 1329-34.

Song, J., et al., *Rapid and effective malaria control in Cambodia through mass administration of artemisinin-piperaquine.* Malar J, 2010. **9**: p. 57.

Smithuis, F., et al., *Effectiveness of five artemisinin combination regimens with or without primaquine in uncomplicated falciparum malaria: an open-label randomised trial.* Lancet Infect Dis, 2010.

Alvarez, G., et al., *Dynamics of Plasmodium falciparum parasitemia regarding combined treatment regimens for acute uncomplicated malaria, Antioquia, Colombia.* Am J Trop Med Hyg, 2010. 83(1): p. 90-6.

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APPENDIX 12: Sample of the label for the imported products

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APPENDIX 13: Letter of authorization from the Trial Sponsor

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APPENDIX 14: Other supporting documents

✓ *Letter of Agreement for Trial to take place*

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INVESTIGATOR'S BROCHURE

Study title: Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda

Study Sponsor: London School of Hygiene and Tropical Medicine

Principal Investigator: Alice C. Eziefula, MBBS, MRCP, MRCPath

Edition Number: 1.0

Release Date: 1st June 2011

TABLE OF CONTENTS

1. PRODUCT:

Primaquine phosphate

2. CHEMICAL NAME:

8-[(4-Amino-1-methylbutyl)amino]-6-methoxyquinoline phosphate

3. MANUFACTURER AND DISTRIBUTOR:

Government Pharmaceutical Industry, Bangkok, Thailand

Contact person: Kanchana Pongsaswat, E-mail: Phung@tropmedres.ac, Wellcome Trust Mahidol Oxford Tropical Medicine Research Unit, 420 6 Rajvithi Road, Bangkok, Thailand 10400

4. FORMULATION:

TheEachdosagetablet containsisexpressed26.3inmgtermsofPrimaquineofthebase.phosphate (equivalent to 15 mg of primaquine base).

5. ACTIVE INGREDIENTS:

Primaquine Phosphate

6. INACTIVE INGREDIENTS:

Calcium Phosphate (tribasic), Lactose, Tapioca starch, Povidone (K-25), Sodium Starch Glycollate, Magnesium stearate, Ethanol (96%)*, Titanium Dioxide, Talcum, Hydroxypropyl Methylcellulose 2910, Polyethelene Glycol 6000, Brilliant Blue Lake, Ponceau 4 R Lake, Sunset Yellow Lake, Tartrazine Lake, Isopropyl Alcohol*, Purified Water*

*evaporated during the process

7. METHOD OF PREPARATION:

Primaquine phosphate is produced according to manufacturing standards of the Thai Government Department of Quality Assurance. The manufacturer's original monograph of the manufacturing process is available in appendix A.

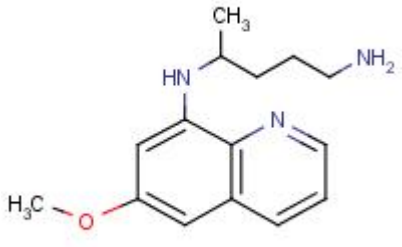
8. QUALITY CONTROL:

In process quality control is performed on granules (moisture content), core tablets (appearance, weight, hardness, disintegration time and friability test) and coated tablets (appearance). Finished

product quality control is conducted for dissolution, uniformity studies and assay for labelled primaquine. Details of the in-process and finished product quality control process from the manufacturer are available in appendix B and appendix C .

9. PHYSICAL AND CHEMICAL PROPERTIES:

Chemical structure:



Molecular Formula: C15-H21-N3-O, 2HPO. Molecular weight: 259.35

Mechanism of action: The exact mechanism of action of primaquine phosphate is unknown. It is likely that it is mediated through the selective generation of oxidative stress in parasitized cells.

Characteristics: An orange-red, crystalline powder; odourless or almost odourless.

Solubilities: Soluble in 16 parts of water, practically insoluble in chloroform and in ether.

Identification[1]:

A. Dissolve 0.1g in 10ml of water, add 2ml of 2M sodium hydroxide and extract with two 20ml quantities of chloroform, reserving the aqueous layer for test C. Wash the chloroform extracts with water, dry with anhydrous sodium sulphate, evaporate to dryness and dissolve the residue in 2ml of chloroform IR. The infra -red absorption spectrum of the resulting solution is concordant with the reference spectrum of primaquine.
B. The light absorption in the range 250 to 300 nm of a 0.003% w/v solution in 0.01M hydrochloric acid exhibits two maxima, at 265nm and 282nm. The absorbance at 265nm is about 0.99 and at 282nm is about 0.98.
C. The aqueous layer obtained in test A, after neutralisation with 2M nitric acid, yields the reactions characteristic of phosphates.

[1] Sources:

1. Government Pharmaceutical Organisation (Thailand) manufacturer's official monographs
2. McEvoy, G.K. (ed.). American Hospital Formulary Service. AHFS Drug Information. American Society of Health-System Pharmacists, Bethesda, MD. 2006., p. 870

Acidity: pH of a 1% w/v solution, 2.5 to 3.5.

Loss on drying: when dried to constant weight, losses not more than 0.5% of its weight. Use 1g.

Assay: dissolve 0.2g in 40ml of anhydrous glacial acetic acid with gentle heating and carry out non-aqueous titration, determining the endpoint potentiometrically. Each ml of 0.1M perchloric acid VS is equivalent to 0.02277g of C15-H21-N3-O, 2HPO.

Stability: Shelf life is 3 years. Long term stability data is available in appendix D.

Storage: Primaquine phosphate tablets should be stored in well-closed, light-resistant containers at a temperature less than 40 deg C, preferably between 15-30 deg C.

Appearance of tablets: Brown, circular biconvex film-coated tablets.

Background 10.INDICATION AND USES:

Primaquine is an old drug, developed in the 1940s and in widespread use (marketed) since the 1960s. It was one of the first synthetic anti-malarials to be developed. It belongs to the 8-aminoquinoline drug class. Other drugs in this class include Tafenoquine and Bulaquine, but these are not yet widely available. *Plasmodium vivax* *Plasmodium falciparum*

Primaquine is effective against the sporozoites of and , and against the hypnozoites of and but it has no effect on the and hypnozoites as anti-relapse therapy. By eliminating these hepatic, tissue *Plasmodium falcip rum.* *P.* (exoerythrocytic) stages of the parasite, primaquine prevents the development of blood stages *vivax* *P. ovale*

(erythrocytic) stages, thus preventing relapses of and . For this is 30mg daily for two weeks, a total dose of 420mg. By eliminating sporozoites,

30mg/day.

Primaquine is also gametocytocidal, that is, it is active against the sexual forms of the *P. falciparum* malaria parasite, the gametocytes. These blood-borne sexual stages, although harmless to humans, are infectious to mosquitoes and are responsible for onward transmission of malaria from human to mosquito. Therefore, by eliminating gametocytes, primaquine acts as a malaria transmission-blocker for falciparum malaria.
Dose for *P. Falciparum* transmission-blocking *P. vivax* 6 mg/kg (30 mg

~~In contrast with the total dose of primaquine~~ base for *P.* , which is base/day) i.e. 420mg, the [2]WHO-recommended dose for transmission-blocking is one *Falciparum*

Primaquine is indicated for the radical cure (prevention of relapse) of vivax malaria. It is used as a prophylactic against all species of malaria. It is used to block transmission of falciparum malaria by eliminating gametocytes.

[2] ~~WHO Malaria Treatment Guideli~~nes 2010

11.PHARMACOKINETICS:

Absorption, distribution and bioavailability: Primaquine is readily absorbed from the gastrointestinal tract. Peak plasma concentration is within 1-4 hours[1-3] and the terminal half life is 4-6 hours[1]. Inter- individual variation in peak plasma concentrations of primaquine has been reported with the same dose of the drug. 24 hr after ingestion the plasma concentration is negligibly low.

Primaquine exhibits extensive tissue distribution [3-4]. About 75% of primaquine in plasma is bound to proteins and high concentrations occur in erythrocytes. Primaquine crosses the placenta but it is uncertain whether significant amounts occur in breast milk.

Metabolism and elimination: Primaquine is extensively metabolized. Several metabolites of primaquine have been identified, but it is unclear which are responsible for the gametocytocidal action and which for its toxic effects. Carboxyprimaquine is the main metabolite [5]and its formation is cytochrome CYP450-dependent [6] . The 5-hydroxylated metabolite has been linked to both therapeutic efficacy and toxicity [7]. Other metabolites have been identified, but their function remains undetermined [8]. Less than 2% of the parent drug, primaquine is excreted in the urine within 24hrs of dosing[1].

Studies that provide pharmacokinetic data on primaquine have been conducted largely in Southeast Asia and Australasia as above. The majority of studies have been on adults. There is a lack of data on the pharmacokinetics and pharmacodynamics of primaquine in African children. Given that primaquine may be deployed in malaria endemic areas in Africa, this data is needed.

12.NON-CLINICAL STUDIES

Organ toxicity: Given at lethal doses, primaquine causes hepatic and cardiac lesions in experimental animals. This has not been demonstrated in humans[9]. Based on its anti-arrhythmic activity in mice, primaquine is predicted to have quinidine like cardiotoxicity.

Mutagenicity: no data available.

Carcinogenicity: no data available.

Reproductive toxicity: no reports are available to associate primaquine with congenital defects.[3]

13.CLINICAL STUDIES

SAFETY STUDIES

1. Tolerability.

The toxicity of primaquine phosphate is dose-dependent. The main toxic effects are gastro-intestinal, methaemoglobinaemia and haemolysis. Gastro-intestinal effects include abdominal cramps, vomiting, burning epigastric pain, diarrhoea. These effects can be avoided if primaquine is

[3] ~~US National Library of Medicine~~ Hazardous substances Data Bank (TOXNET)

administered with food or a small snack[10]. Methaemoglobinaemia can cause cyanosis. In healthy subjects given primaquine for prophylaxis, primaquine elevates methaemoglobin levels by about 4% [1]. The maximum reported rise was 13% in Indonesia[11]. Methaemoglobin levels less than 20% are typically tolerated without signs or symptoms [12]. In individuals with nicotinamide adenine dinucleotide methemoglobin reductase deficiency, methaemoglobinaemia may be clinically significant (cyanosis and shortness of breath).

In individuals with G6PD deficiency, primaquine causes transient, dose-dependent haemolysis. It is likely that this is due to the effect of one of primaquine's metabolites and that it is mediated through oxidative stress, but the exact mechanism is as yet unknown. In a population, the risk of haemolysis with primaquine corresponds with the frequency of the defective gene the degree of G6PD enzyme dysfunction it codes for. In a mass screen and treatment programme in Tanzania, in asymptomatic parasitized children aged 1 to 12 years [13], the mean change in haemoglobin after a single dose of 0.75mg/kg primaquine in combination with sulphadoxine-pyrimethamine artesunate treatment was - 0.58g/dL. In G6PD heterozygotes, the mean change in haemoglobin was - 1.6g/dL and in homozygote/ hemizygote deficient children, the mean change in haemoglobin was -2.5g/dL. One child had severe anaemia by haemoglobin measurement (4.8g/dL) but their G6PD status was not reported. No child required a blood transfusion.

In a Tanzanian study where 0.75mg/kg primaquine was given to children aged 3 to 15 years with clinical malaria [14], the mean fall in haemoglobin was 5.2% from enrolment value and this was found on day 7 after primaquine was administered. No child required a blood transfusion and no child had symptomatic anaemia.

Other reported toxic manifestations include cardiovascular disturbances (ventricular dysrhythmias, and hypertension have been reported on rare occasions of chronic poisoning [4], headache, confusion, interference with visual accommodation, pruritis and leucocytosis or leucopenia. Following a course of primaquine for vivax malaria (30mg for 14 days), there is a single case report of depression and psychosis[15] and a single case report of confusion and hallucinations [16] in the literature.

2. Safety guidelines: Discontinue the use of primaquine phosphate promptly if signs suggestive of hemolytic anaemia occur (darkening of the urine, marked fall of haemoglobin or erythrocytic count).[5]
3. Contra-indications[6]: The balance of risk and benefit should be considered when primaquine is administered under the following conditions: acutely ill patients suffering from systemic disease manifested by tendency to granulocytopenia, such as rheumatoid arthritis and lupus erythematosus. The drug is also contraindicated in patients receiving concurrently other potentially haemolytic drugs or depressants of myeloid elements of the bone marrow

[4] ~~US~~US ~~National~~FoodandLibrary~~Library~~DrugAssociation,~~ofMedicine~~PrimaquineHazardous drugsubstancessafety information,DataBank(TOXNET)2008..

[5]

[6] International Programme on Chemical Safety; Poisons Information Monograph: Primaquine Phosphate (PIM 434) (1994)

(such as antineoplastic agents, colchicine, gold salts, penicillamine, phenylbutazone, quinacrine).
Because quinacrine hydrochloride appears to potentiate the toxicity of antimalarial compounds which are structurally related to primaquine, the use of quinacrine in patients receiving primaquine is contraindicated. Similarly, primaquine should not be administered to patients who have received quinacrine within 3 months, as toxicity is increased.
Concurrent use of primaquine with bone marrow depressants may increase the risk of leukopenia. If concurrent use is essential, close observation for myelotoxicity should be considered.

4. Use in special populations:
Pregnant women: Primaquine is contra-indicated in pregnancy because it is not possible to ascertain the G6PD status of the foetus and hence its risk of haemolysis.

Lactating women[7]: There is no available data on the excretion of primaquine into breast milk. Problems in man have not been documented.

Geriatric use[8]: There is insufficient data in subjects aged 65 and over to determine whether their response to primaquine differs from younger subjects. Reported clinical experience has not identified differences in responses between the elderly and younger patients. In general, dose selection for an elderly patient should be cautious, usually starting at the low end of the dosing range, reflecting the greater frequency of decreased hepatic, renal, or cardiac function, and of concomitant disease or other drug therapy.

[7] International Programme on Chemical Safety; Poisons Information Monograph: Primaquine Phosphate (PIM 434)

[8] US Food and Drug Association, Primaquine drug safety information, 2008.8(1994)

EFFICACY STUDIES

Efficacy trial data and summary table

of publication	primaquine, day of treatment	group			effect of primaquine
[17]	single dose, day 2	years	uncomplicated falciparum malaria		circulation time reduced from 286 to 6.3 days (p<0.001)
[14]	single dose, day 2	years	uncomplicated falciparum malaria		prevalence reduced from 62.7% to 3.9% (p<0.001)
[18]	single dose, day 2	to adult	administration, asymptomatic falciparum malaria		difference in gametocyte prevalence on day 7 or day 14 post treatment
[19]	mg/kg/day for 7 days and 0.5 mg/kg/day for 7 days		uncomplicated falciparum malaria	Artesunate	odds ratio of 0 .42 (0.20 to 0.83); =0.009 No significant *P* difference between primaquine dosage groups
[20]	single dose, day 0	to adult	uncomplicated falciparum malaria	AA, AL, DP*	carriage rate ratio 11·9 (95% CI 7·4–20·5; *P* = 0.0001).
[21]	dose	to adult	uncomplicated falciparum malaria	Mefloquine, plus 5 other regimes (not all ACT)	non-primaquine-containing regimens, reduction in time to gametocyte clearance: 1 week

*AM-F=artesunate–mefloquine fixed-dose combination. AM-L=artesunate–mefloquine loose tablets.

AA=artesunate–amodiaquine. AL=artemether–lumefantrine. DP=dihydroartemisinin–piperaquine

A recent study conducted in Tanzania[17] in asymptomatic parasitized children demonstrated a dramatic reduction of gametocyte circulation time with primaquine treatment from 28.6 days in the absence of primaquine (with ACT alone) to 6.3 days with primaquine.

Primaquine reduced gametocytaemia significantly at days 4, 7, 14 and 28 post treatment in a Tanzanian study[14] comparing ACT with or without primaquine in children with uncomplicated clinical malaria. Here, the prevalence of gametocytes on day 14 after treatment was reduced from 62.7% to 3.9%.

In Thailand[19], patients presenting with uncomplicated malaria in Bangkok had reduced gametocyte clearance times when primaquine was added to all drug combinations. Primaquine reduced gametocyte clearance with an odds ratio of 0.42 (0.20 to 0.83); P =0.009.

In a recent study in Burma[20], 808 participants were randomized to receive ACT plus primaquine or ACT alone. Gametocyte carriage was substantially reduced by the addition of primaquine (rate ratio 11·9 (95% CI 7·4–20·5; 0.0001). There was an overall increase in haemoglobin during

primaquine group (0.75 g/dL 1.04 g/dL; =0.036; mean difference 0.295 g/dL; 95% CI 0.199–

there were no severe adverse events. The only adverse event attributable to primaquine was abdominal pain. This is a known side effect and is reduced by administration with food[10].

In Colombia [21], investigators found a disappearance of gametocytes one week earlier when PQ

was added to an artemisinin-containing regimen. *Plasmodium vivax*

14.MARKETING EXPERIENCE:

PQ was developed by the US Army[9] in the 1940s for radical cure of . It was used to prevent relapse of vivax malaria in servicemen repatriated to the United States. Primaquine has been on the WHO essential drugs list since 1977. Currently, no Cochrane reviews are completed on the use of primaquine for gametocytocidal action. Observations that primaquine is effectively gametocytocidal were recorded in Bulletin of the WHO in 1961[22]. The use of single-dose

primaquine as a gametocytocidal drug has[10] been advised by the WHO since the 1970s without the for radical cure of for patients with G6PD. deficiency and caution upon prescription of primaquine in the context of acute malaria In 2008 and 2010, the WHO advised the use of primaquine as a gametocytocidal drug for malaria elimination and control in combination with ACTs (artemisinin combination therapies). Several nations in Southeast Asia and South America, including China, Indonesia, Thailand, India, Sri Lanka and Colombia advise primaquine for gametocyte clearance. It has not yet been used widely for this purpose in African countries.

Primaquine is listed on the National Drug Authority (NDA) register of approved medications in

Uganda under drug registration number 1256/06/97.

[9] ~~Edgcomb, J.H. et al. (1950) Prima~~quine,SN13272,a new curative agent in vivax malaria; a preliminary report. J.[0] Nat. Malaria Assoc. 9, 285–292

World Health Organization (2000) The Use of Antimalarial Drugs: Report of a WHO Informal Consultation,

13-17 November 2000 (WHO/ CDS/RBM/2001.33),World Health Organization

15. REFERENCES

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2. EDWARDS, G., ET AL., *INTERACTIONS AMONG PRIMAQUINE, MALARIA INFECTION AND OTHER ANTIMALARIALS IN THAI SUBJECTS.* BR J CLIN PHARMACOL, 1993. 35(2): P. 193-8.
3. ELMES, N.J., ET AL., *LACK OF SEX EFFECT ON THE PHARMACOKINETICS OF PRIMAQUINE.* AM J TROP MED HYG, 2006. 74(6): P. 951-2.
4. MIHALY, G.W., ET AL., *PHARMACOKINETICS OF PRIMAQUINE IN MAN. I. STUDIES OF THE ABSOLUTE BIOAVAILABILITY AND EFFECTS OF DOSE SIZE.* BR J CLIN PHARMACOL, 1985. 19(6): P. 745-50.
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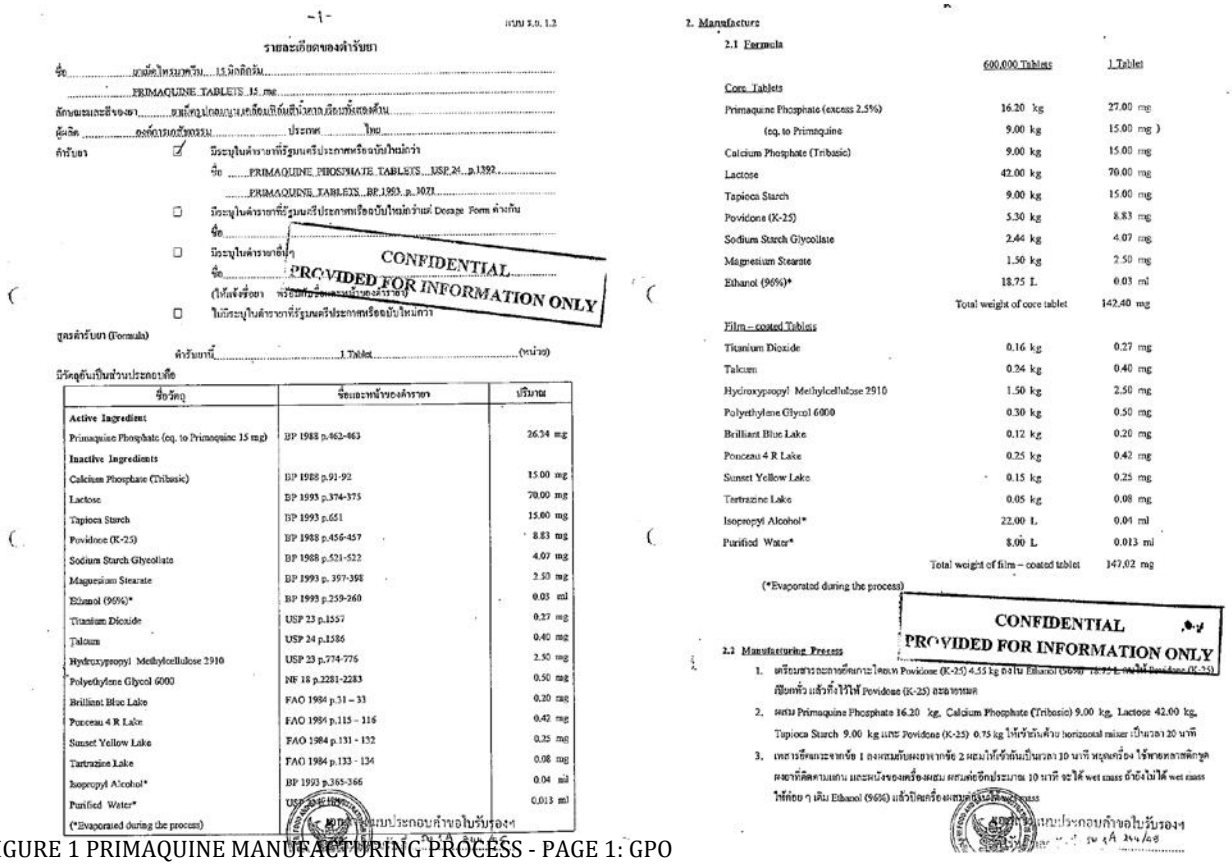
APPENDICES

APPENDIX A: MANUFACTURING PROCESS (THAI).

English version is not available. Chemicals are in English.



-1-

แบบ F.D. 1.2

รายละเอียดของตำรับยา

ชื่อ ยาเม็ดไพรมาควิน 15 มิลลิกรัม

PRIMAQUINE TABLETS 15 mg

ลักษณะและชื่อของยา ยาเม็ดรูปอัดบานงเคลือบเยื่อสีน้ำตาลเยื่อบนทั้งสองด้าน

ผู้ผลิต องค์การเภสัชกรรม ประเทศ ไทย

ตำรับยา ☑ มีระบุในตำรายาที่รับรองหรือฉบับใหม่กว่า

ชื่อ PRIMAQUINE PHOSPHATE TABLETS USP 24 p.1392

PRIMAQUINE TABLETS BP 1993 p. 1073

☐ มีระบุในตำรายาที่รับรองหรือฉบับใหม่กว่าแต่ Dosage Form ต่างกัน

ชื่อ

☐ มีระบุในตำรายาอื่นๆ

ชื่อ

(โปรดระบุชื่อยา หรือสิ่งที่ระบุในตำรายา)

☐ ไม่มีระบุในตำรายาที่รับรองหรือฉบับใหม่กว่า

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สูตรตำรับยา (Formula)

ตำรับยานี้ 1 Tablet (หน่วย)

มีวัตถุดิบเป็นส่วนประกอบดังนี้

ชื่อวัตถุ	ชื่อและหน้าของตำรายา	ปริมาณ
Active Ingredient		
Primaquine Phosphate (eq. to Primaquine 15 mg)	BP 1988 p.462-463	26.34 mg
Inactive Ingredients		
Calcium Phosphate (Tribasic)	BP 1988 p.91-92	15.00 mg
Lactose	BP 1993 p.374-375	70.00 mg
Tapioca Starch	BP 1993 p.651	15.00 mg
Povidone (K-25)	BP 1988 p.456-457	8.83 mg
Sodium Starch Glycollate	BP 1988 p.521-522	4.07 mg
Magnesium Stearate	BP 1993 p. 397-398	2.50 mg
Ethanol (96%)*	BP 1993 p.259-260	0.03 ml
Titanium Dioxide	USP 23 p.1557	0.27 mg
Talcum	USP 24 p.1586	0.40 mg
Hydroxypropyl Methylcellulose 2910	USP 23 p.774-776	2.50 mg
Polyethylene Glycol 6000	NF 18 p.2281-2283	0.50 mg
Brilliant Blue Lake	FAO 1984 p.31 – 33	0.20 mg
Ponceau 4 R Lake	FAO 1984 p.115 – 116	0.42 mg
Sunset Yellow Lake	FAO 1984 p.131 - 132	0.25 mg
Tartrazine Lake	FAO 1984 p.133 - 134	0.08 mg
Isopropyl Alcohol*	BP 1993 p.365-366	0.04 ml
Purified Water*	USP 23 p.1555	0.013 ml

(*Evaporated during the process)

2. Manufacture

2.1 Formula

	600,000 Tablets	1 Tablet
Core Tablets		
Primaquine Phosphate (excess 2.5%)	16.20 kg	27.00 mg
(eq. to Primaquine	9.00 kg	15.00 mg)
Calcium Phosphate (Tribasic)	9.00 kg	15.00 mg
Lactose	42.00 kg	70.00 mg
Tapioca Starch	9.00 kg	15.00 mg
Povidone (K-25)	5.30 kg	8.83 mg
Sodium Starch Glycollate	2.44 kg	4.07 mg
Magnesium Stearate	1.50 kg	2.50 mg
Ethanol (96%)*	18.75 L	0.03 ml
	Total weight of core tablet	142.40 mg
Film – coated Tablets		
Titanium Dioxide	0.16 kg	0.27 mg
Talcum	0.24 kg	0.40 mg
Hydroxypropyl Methylcellulose 2910	1.50 kg	2.50 mg
Polyethylene Glycol 6000	0.30 kg	0.50 mg
Brilliant Blue Lake	0.12 kg	0.20 mg
Ponceau 4 R Lake	0.25 kg	0.42 mg
Sunset Yellow Lake	0.15 kg	0.25 mg
Tartrazine Lake	0.05 kg	0.08 mg
Isopropyl Alcohol*	22.00 L	0.04 ml
Purified Water*	8.00 L	0.013 ml
	Total weight of film – coated tablet	147.02 mg

(*Evaporated during the process)

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2.2 Manufacturing Process

1. เตรียมสารละลายเอทิลแอลกอฮอล์ Povidone (K-25) 4.55 kg ใน Ethanol (96%) 18.72 L จนได้ Povidone (K-25)
เปียกทั่วแล้วทิ้งไว้ให้ Povidone (K-25) ละลายหมด
2. ผสม Primaquine Phosphate 16.20 kg, Calcium Phosphate (Tribasic) 9.00 kg, Lactose 42.00 kg,
Tapioca Starch 9.00 kg และ Povidone (K-25) 0.75 kg ให้เข้ากันด้วย horizontal mixer เป็นเวลา 20 นาที
3. เทสารที่ผสมจากข้อ 1 ลงผสมกับผงยาจากข้อ 2 ผสมให้เข้ากันเป็นเวลา 10 นาที หยุดเครื่อง ใช้พายพลาสติกขูด
ผงยาที่ติดตามผนัง และหน้าของเครื่องผสม ผสมต่อยีกประมาณ 10 นาที จะได้ wet mass ถ้ายังไม่ได้ wet mass
ให้ค่อย ๆ เติม Ethanol (96%) แล้วปิดเครื่องผสมทิ้งไว้ประมาณ wet mass

FIGURE 1 PRIMAQUINE MANUFACTURING PROCESS - PAGE 1: GPO

4. นำ wet mass ที่ได้ใส่ลงในถาดอบยา เกลี่ยให้มีความหนา 2-3 เซนติเมตร แล้วผึ่งข้ามคืนเพื่อให้หมาด
5. นำ wet mass ที่ได้ไปแร่งด้วย oscillating granulator ผ่าน sieve ขนาด 14 mesh ใส่ลงในถาดอบยา เกลี่ยให้มีความหนา 2 – 3 เซนติเมตร แล้วทิ้งข้ามคืนเพื่อให้ Ethanol (96%) บางส่วนระเหยออก
6. นำ granules เข้าอบใน tray type dryer ที่อุณหภูมิ (60±5) °C นานประมาณ 1 ชั่วโมง เพื่อให้เหลือปริมาณความชื้น ประมาณ 3.0% แต่ไม่เกิน 4.0%
7. นำ dried granules ที่อบแห้งแล้วไปแร่งด้วย oscillating granulator ผ่าน sieve ขนาด 16 mesh
8. แบ่ง granules มาประมาณ 10 กรัม เพื่อหาปริมาณความชื้นด้วยเครื่องหาปริมาณความชื้น (กำหนดปริมาณความชื้นประมาณ 3.0% แต่ไม่เกิน 4.0%)
9. ชั่งและบันทึกน้ำหนัก dried granules
10. ชั่ง Magnesium Stearate 1.50 kg และ Sodium Starch Glycollate 2.44 kg แล้วนำไปผ่านแร่งมือขนาด 30 mesh
11. ผสม dried granules กับผงยาในข้อ 11 ด้วย cubic mixer นานประมาณ 5 นาที
12. ชั่งและบันทึกน้ำหนัก granules ที่ได้
13. นำ granules เข้าเครื่องตอกเม็ดยา โดยปรับน้ำหนักเม็ดยา 10 เม็ด ให้ได้น้ำหนักเท่ากับ 1.424 กรัม และให้ได้คุณสมบัติอื่นๆตาม Specification and Testing Standard for Finished Product
14. ชั่งและบันทึกน้ำหนักเม็ดยาสำเร็จรูปที่ได้
15. ชั่ง Brilliant Bule Lake 0.12 kg , Ponceau 4R Lake 0.25 kg, Sunset Yellow Lake 0.15 kg, Tartrazine Lake 0.05 kg, Titanium Dioxide 0.16 kg และ Talcum 0.24 kg
16. ตวง Alcohol Isopropyl จำนวน 10 ลิตร ใส่ในภาชนะที่เหมาะสม
17. ค่อย ๆ โปรยผงยาจากข้อ 15 ลงในข้อ 16 พร้อมกับคนด้วยพายสเตนเลสเพื่อไม่ได้ผงยารวมตัวกันเป็นก้อน
18. ชั่ง Hydroxypropyl Methylcellulose 2910 จำนวน 1.50 kg
19. ตวง Alcohol Isopropyl จำนวน 12 ลิตร ใส่ในภาชนะที่เหมาะสม
20. โปรยผงยาจากข้อ 18 ลงในข้อ 19 แล้วใช้ homogenizer ปั่นให้ได้ suspension ใช้เวลาประมาณ 20 นาที
21. ชั่ง Polyethylene Glycol 6000 0.30 kg แล้วละลายลงในน้ำจำนวน 8 ลิตร
22. เทสารละลาย จากข้อ 21 ลงในข้อ 20 แล้วใช้ homogenizer ปั่นต่อให้ได้ suspension ใส
23. เทสารละลายในข้อ 17 ลงในข้อ 22 แล้วใช้ homogenizer ปั่นให้เข้ากันอีกประมาณ 30 นาที จนเป็นเนื้อเดียวกัน
24. นำไปกรองด้วย sieve ขนาด 100 mesh ใส่ลงใน stock tank เพื่อทำการเคลือบ แล้วทำการเคลือบเม็ดยาที่ได้
25. ชั่งและบันทึกน้ำหนักเม็ดยาสำเร็จรูปที่ได้
26. ฝ่ายประกันคุณภาพสุ่มเก็บตัวอย่างเพื่อตรวจสอบคุณภาพ

FIGURE 2 PRIMAQUINE MANUFACTURING PROCESS - PAGE 2: GPO

APPENDIX B: IN-PROCESS QUALITY CONTROL STANDARDS FROM MANUFACTURER GPO

3.2 Inprocess Control

Test	Requirement
Granules	
Moisture Content	3.0 – 4.0 %
% Primaquine	10.35 – 10.70 % w/w
Core Tablets	
Appearance	Orange, circular, biconvex, compressed tablets
Weight per 10 tablets	1.424 g (±) 5%
Range of Mean Weight	142.40 mg (±) 5%
Hardness	Not less than 3.0 kp
Disintegration Time	Not more than 15 minutes
Friability Test	Not more than 0.6% w/w
Coated Tablets	
Appearance	Brown, circular, biconvex, film – coated tablets

FIGURE 3 PRIMAQUINE IN-PROCESS QUALITY CONTROL: GPO

- MANUFACTURER

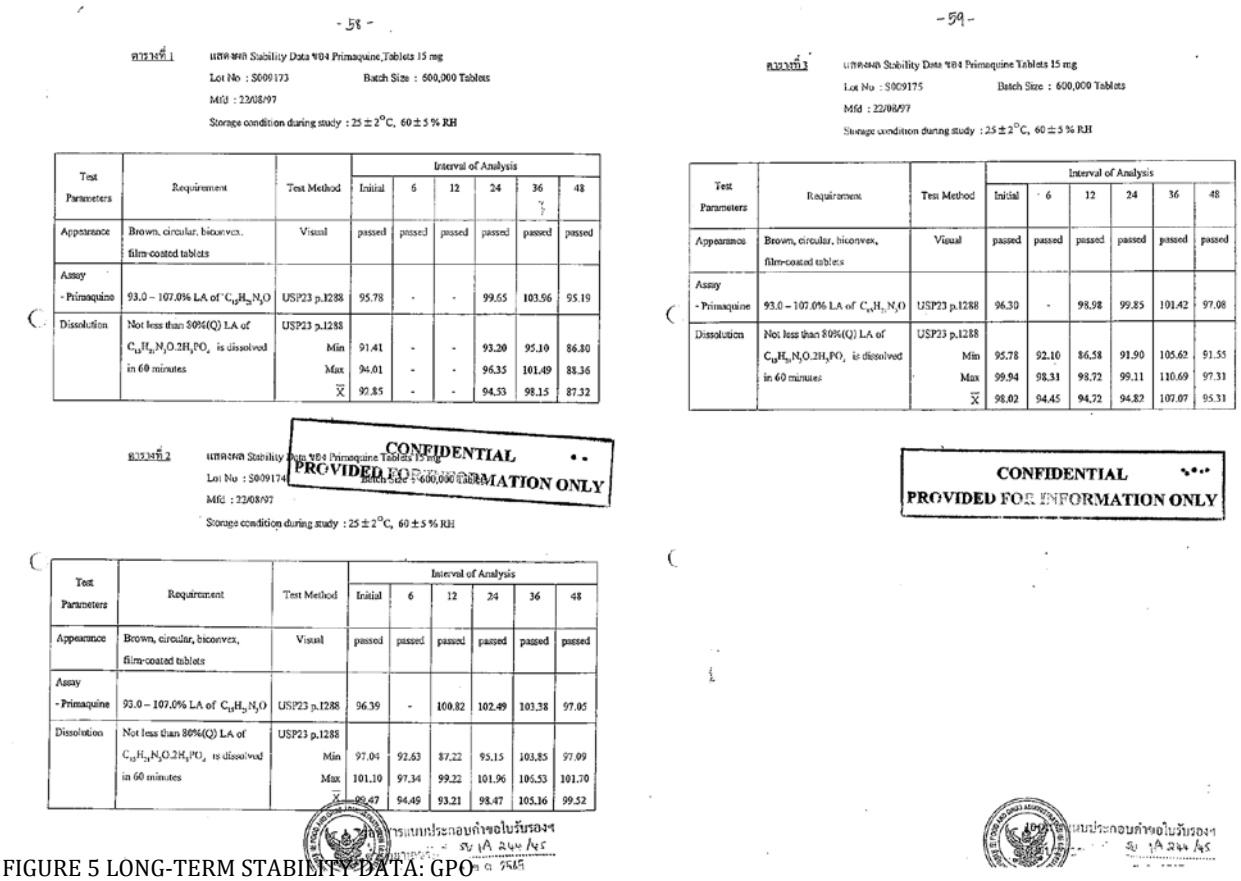
3.3 FINISHED PRODUCT SPECIFICATION AND CONTROL METHOD

Product Name : PRIMAQUINE TABLETS 15 mg

Appearance : ยาเม็ดรูปกลมนูน เคลือบฟิล์มสีน้ำตาล เรียบทั้งสองด้าน

TEST	REQUIREMENT	METHOD
Identification	Positive	BP 1993 p.1071
Dissolution	Not less than 80% (Q) of the labeled amount of $C_{15}H_{21}N_3O.2H_3PO_4$ is dissolved in 60 minutes	USP 24 p.1392
Uniformity of dosage units by content uniformity	85.0 – 115.0 % LA of $C_{15}H_{21}N_3O$, % RSD ≤ 6.0 %	USP 24 p.2000
Assay - Primaquine	93.0 – 107.0% of the labeled amount of $C_{15}H_{21}N_3O$	USP 24 p.1392
Shelf - life	3 years	
Storage Condition	เก็บที่อุณหภูมิไม่เกิน 25 °C	

FIGURE 4PRIMAQUINE FINISHED PRODUCT SPECIFICATIONS: GPO



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ตารางที่ 1 แสดงผล Stability Data ของ Primaquine,Tablets 15 mg

Lot No : S009173 Batch Size : 600,000 Tablets

Mfd : 22/08/97

Storage condition during study : 25 ± 2°C, 60 ± 5 % RH

Test Parameters	Requirement	Test Method	Interval of Analysis					
			Initial	6	12	24	36	48
Appearance	Brown, circular, biconvex, film-coated tablets	Visual	passed	passed	passed	passed	passed	passed
Assay - Primaquine	93.0 – 107.0% LA of $C_{15}H_{21}N_3O$	USP23 p.1288	95.78	-	-	99.65	103.96	95.19
Dissolution	Not less than 80%(Q) LA of $C_{15}H_{21}N_3O.2H_3PO_4$ is dissolved in 60 minutes	USP23 p.1288 Min	91.41	-	-	93.20	95.10	86.80
		Max	94.01	-	-	96.35	101.49	88.36
		$\bar{X}$	92.85	-	-	94.53	98.15	87.32

ตารางที่ 2 แสดงผล Stability Data ของ Primaquine Tablets 15 mg

Lot No : S009174 Batch Size : 600,000 Tablets

Mfd : 22/08/97

Storage condition during study : 25 ± 2°C, 60 ± 5 % RH

Test Parameters	Requirement	Test Method	Interval of Analysis					
			Initial	6	12	24	36	48
Appearance	Brown, circular, biconvex, film-coated tablets	Visual	passed	passed	passed	passed	passed	passed
Assay - Primaquine	93.0 – 107.0% LA of $C_{15}H_{21}N_3O$	USP23 p.1288	96.39	-	100.82	102.49	103.38	97.05
Dissolution	Not less than 80%(Q) LA of $C_{15}H_{21}N_3O.2H_3PO_4$ is dissolved in 60 minutes	USP23 p.1288 Min	97.04	92.63	87.22	95.15	103.85	97.09
		Max	101.10	97.34	99.22	101.96	106.53	101.70
		$\bar{X}$	99.47	94.49	93.21	98.47	105.16	99.52

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ตารางที่ 3 แสดงผล Stability Data ของ Primaquine Tablets 15 mg

Lot No : S009175 Batch Size : 600,000 Tablets

Mfd : 22/08/97

Storage condition during study : 25 ± 2°C, 60 ± 5 % RH

Test Parameters	Requirement	Test Method	Interval of Analysis					
			Initial	6	12	24	36	48
Appearance	Brown, circular, biconvex, film-coated tablets	Visual	passed	passed	passed	passed	passed	passed
Assay - Primaquine	93.0 – 107.0% LA of $C_{15}H_{21}N_3O$	USP23 p.1288	96.30	-	98.98	99.85	101.42	97.08
Dissolution	Not less than 80%(Q) LA of $C_{15}H_{21}N_3O.2H_3PO_4$ is dissolved in 60 minutes	USP23 p.1288 Min	95.78	92.10	86.58	91.90	105.62	91.55
		Max	99.94	98.31	98.72	99.11	110.69	97.31
		$\bar{X}$	98.02	94.45	94.72	94.82	107.07	95.31

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FIGURE 5 LONG-TERM STABILITY DATA: GPO

APPENDIX C

1) *Data Safety Monitoring Board reporting sheet*
2) *DSMB statement on un-blinding*

DSMB shell report tables

Study Title:	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda
Source of funding:	The Wellcome Trust
Sponsor:	London School of Hygiene and Tropical Medicine
Site of Research:	Walukuba Health Centre IV, Jinja, Uganda
Principal Investigator:	Dr. Chi Eziefula

1. Enrolment for each site

Month of study	Treatment arm (number enrolled)				
	A	B	C	D	TOTAL
1					
2					
3					
4					
5					
6					
7 (until sample size complete)					

2. Withdrawal reasons by trial arm

Withdrawal reason	Treatment arm				***Total***
	A	B	C	D	
Moved from study area	N (%)	N (%)	N (%)	N (%)	N (%)
Concern regarding study drug					N (%)
No reason given, just wants to withdraw					N (%)
Adverse event prompted withdrawal by participant					N (%)
Died					N (%)
Vomiting PQ/placebo > X2					N (%)
Study physician decision to withdraw					N (%)
Other					N (%)
Total	**n (100%)**	**n (100%)**	**n (100%)**	**n (100%)**	**n (100%)**

3. Loss to follow-up

Month of study	Treatment arm number (%) lost to follow-up				
	A	B	C	D	TOTAL
1					
2					
3					
4					
5					
6					
7 (until sample size complete)					

4. Follow up numbers at 1, 2, 3, 4, 7, 10, 14, 21 and 28 days

	1	*2*	*3*	*4*	*7*	*10*	*14*	*21*	*28*
Group A									
Attended	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Defaulted[1]									
Withdrew[2]									
Total	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***
Group B									
Attended	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Defaulted[1]									
Withdrew[2]									
Total	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***
Group C									
Attended	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Defaulted[1]									
Withdrew[2]									
Total	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***
Group D									
Attended	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Defaulted[1]									

Withdrew[2]									
Total	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***	***N (100%)***
All groups									
Attended	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Defaulted[1]									
Withdrew[2]									
Grand Total (n=)	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***

1 Participant did not attend visit but has not formally withdrawn from the study.

2 Cumulative withdrawals. Participants who have formally dropped out of the study for any reason (includes deaths)

5. Missing Data/ incomplete follow up

	1	*2*	*3*	*4*	*7*	*10*	*14*	*21*	*28*
Group A									
Missing Hb	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Missing L6 sample									
Missing 903 paper									
Missing Clinical review									
Missing parasitaemia									
Missing G6PD Day 14 ELISA									
Group B									
Missing Hb	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Missing L6 sample									
Missing 903 paper									
Missing Clinical review									
Missing parasitaemia									
Missing G6PD Day 14 ELISA									
Group C									
Missing Hb	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Missing L6 sample									
Missing 903 paper									

Missing Clinical review									
Missing parasitaemia									
Missing G6PD Day 14 ELISA									
Group D	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Missing Hb									
Missing L6 sample									
Missing 903 paper									
Missing Clinical review									
Missing parasitaemia									
Missing G6PD Day 14 ELISA									
All groups	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
Missing Hb									
Missing L6 sample									
Missing 903 paper									
Missing Clinical review									
Missing parasitaemia									
Missing G6PD Day 14 ELISA									

6. Parameters during follow up for this month

	1	*2*	*3*	*4*	*7*	*10*	*14*	*21*	*28*
Haemoglobin <5 (g/dL)	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
GROUP A									
GROUP B									
GROUP C									
GROUP D									
TOTAL	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***
Requirement for transfusion[1]	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
GROUP A									

[1] Number of units transfused will be available

GROUP B									
GROUP C									
GROUP D									
TOTAL	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***
Black urine									
GROUP A	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
GROUP B									
GROUP C									
GROUP D									
TOTAL	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***
Severe adverse event[2]									
GROUP A	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
GROUP B									
GROUP C									
GROUP D									
TOTAL	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***
Gastrointestinal adverse events ≥ grade 3									
GROUP A									
GROUP B									
GROUP C									
GROUP D									
TOTAL	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***	***N (%)***

[2] Sign, symptom or laboratory value of severity grade 3 or above (See Appendix R: "Severity grading of adverse events")

Protocol Title:	Evaluation of the efficacy and safety of primaquine for clearance of gametocytes in uncomplicated falciparum malaria in Uganda
Site of Research:	Walukuba Health Centre IV, Jinja, Uganda
Principal Investigators:	Dr. Chi Eziefula
Date:	27 May 2011

DSMB statement on trial unblinding and target sample size

Since the start of this trial, there has been a shift in the global recognition of the potential role of primaquine in malaria elimination. Primaquine dose-finding for transmission-blocking has become an international priority. In the last week of september 2012, the WHO Malaria Policy Advisory Group announced that they have changed the WHO guidelines to recommend a lower dose of primaquine (0.25mg/kg) based on historical evidence. No formal dose-finding trial is available in the literature and the WHO has indicated that they are aware of our trial and others that have not yet started and they suggest they may well change the guidelines again, accordingly.

The trial investigators are due to give an oral presentation at the 61st annual American Society for Tropical Medicine & Hygiene meeting in Atlanta on 12th November 2012. The WHO Global Malaria Programme have approached the trial investigators to ask whether they (Rob Newman) can present a single slide at the end of our presentation, giving the updated WHO recommendations.

The investigators feel it is going to be important to have data to present at ASTMH. However, given the lower than expected prevalence of malaria in Jinja this year, recruitment is not complete. This means it will not be possible to present the full results.

The blinded analysis has begun and we would be very keen to learn whether you would consider that we **unblind** a proportion of the data in order that we can make a presentation of preliminary analyses.

The trial DSMB was consulted on this matter on 18th October 2012. There was consensus that the decision to unblind for a preliminary analysis is reasonable given these developments. The final results of the trial will be submitted for publication in a peer reviewed publication once recruitment concludes.

The DSMB reviewed the trial progress with regards recruitment and agreed that the rate of loss to follow up (5% rather than the estimated 10%) would imply that the target sample size should be 460 rather than 480 participants.

The signatures below (each in separate documents) confirm the DSMB members' agreement with this statement.

Name	Position	Signature	Date
Grant Dorsey	Chair of DSMB		
Sophie Namasopo	DSMB member		
Jim Todd	DSMB member		
Justus Byarugaba	DSMB member		

APPENDIX D

RESEARCH PAPER 6:

G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD tests

MALARIA JOURNAL

MEETINGREPORT Open Access

G6PD testing in support of treatment and elimination of malaria: recommendations for evaluation of G6PD tests

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Abstract

Malaria elimination will be possible only with serious attempts to address asymptomatic infection and chronic infection by both Plasmodium falciparum and Plasmodium vivax. Currently available drugs that can completely clear a human of P. vivax (known as “radical cure”), and that can reduce transmission of malaria parasites, are those in the 8-aminoquinoline drug family, such as primaquine. Unfortunately, people with glucose-6-phosphate dehydrogenase (G6PD) deficiency risk having severe adverse reactions if exposed to these drugs at certain doses. G6PD deficiency is the most common human enzyme defect, affecting approximately 400 million people worldwide.

Scaling up radical cure regimens will require testing for G6PD deficiency, at two levels: 1) the individual level to ensure safe case management, and 2) the population level to understand the risk in the local population to guide Plasmodium vivax treatment policy. Several technical and operational knowledge gaps must be addressed to expand access to G6PD deficiency testing and to ensure that a patient’s G6PD status is known before deciding to administer an 8-aminoquinoline-based drug.

In this report from a stakeholder meeting held in Thailand on October 4 and 5, 2012, G6PD testing in support of radical cure is discussed in detail. The focus is on challenges to the development and evaluation of G6PD diagnostic tests, and on challenges related to the operational aspects of implementing G6PD testing in support of radical cure. The report also describes recommendations for evaluation of diagnostic tests for G6PD deficiency in support of radical cure.

Goals of the G6PD workshop

In October 2012, a workshop in Bangkok, Thailand, brought together researchers, diagnostic test developers, drug developers, National Malaria Control Programme (NMCP) representatives, development partners and donors to discuss priority issues related to malaria treatment [1]. The workshop built upon two previous meetings: a March 2012 meeting in London on the rationale for short-course primaquine in Africa to interrupt malaria transmission [2] and a May 2012 workshop on glucose-6-phosphate dehydrogenase (G6PD) deficiency that was held in South Korea as part of the Asia Pacific Malaria Elimination Network Vivax Working Group annual meeting [3,4]. The Bangkok workshop provided a forum for discussing the knowledge gaps, barriers, and research questions that must be addressed to support broader availability, adoption, and access to G6PD testing in support of radical cure of Plasmodium vivax.

The goals of the Bangkok workshop were to:

1. Identify technical research priorities to support development of appropriate G6PD testing technologies and strategies in support of P. vivax radical cure.
2. Define use case scenarios or malaria treatment-seeking behaviours that a G6PD test or test result must support.

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3. Identify operational research priorities to support implementation of appropriate G6PD testing technologies and strategies.

Primaquine can be used at low doses as a malaria gametocytocidal to block the transmission of the parasite to the mosquito, or it can be used at higher doses in longer regimens for radical cure of P. vivax infection. The workshop focused on the use of G6PD testing in support of radical cure. The agenda and selected presentations are available online [1].

Background and context

G6PD deficiency is the most common human enzyme defect, affecting more than 400 million people worldwide [5]. Several recent reviews have explored the relationship between malaria and G6PD deficiency [4,6-8]. The meeting focused on topics relevant to developing and evaluating in vitro diagnostic tests for G6PD activity.

Glucose-6-phosphate dehydrogenase

G6PD is a critical housekeeping enzyme in red blood cells that supports protective systems against oxidative challenge by producing the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). The gene for the G6PD enzyme is spread over 18.5 Kb and 13 exons on the X chromosome and encodes for a 59 KDa polypeptide. The enzyme is active as a dimer or dimer of dimers configuration. G6PD deficiency is manifested in people with reduced levels of intra-erythrocyte G6PD activity arising typically from mutations in the G6PD gene that impact the stability of the enzyme.

Results from several studies suggest that G6PD deficiency may confer some protection not only against severe malaria but also against non-severe disease [9-11]. Indeed, G6PD deficiency prevalence overlaps significantly with current and historical malaria endemicity [12]. Within these populations, the protection conferred by G6PD deficiency may result in a reduced prevalence of G6PD deficiency among malarial patients as compared to the general population [9-11].

Definition of G6PD activity

One International Unit (U) is the amount of G6PD activity that will convert 1 micromole of NADP + per minute under predetermined substrate and reaction conditions [13]. Activity may be expressed in either a standard number of cells ($U/10^{12}$ red blood cells) or amount of haemoglobin (U/g Hb). G6PD activity is typically deter-mined by measuring G6PD activity in lysate from a whole blood specimen or a red blood cell preparation from a specimen. G6PD deficiency is defined as a less-than-normal level of G6PD enzyme activity in a blood specimen.

Almost 400 allelic variants in the G6PD gene have been recorded [8,14,15]. The variants known to result in G6PD deficiency tend to affect the stability of the enzyme rather than the catalytic activity of the enzyme [7,8,14,15]. G6PD variants are categorized based on the severity of the G6PD deficiency they cause. Class 1 variants cause congenital non-spherocytic haemolytic anaemia. Class 2 variants cause severe enzyme deficiency (less than 10% of normal). Class 3 variants cause moderate to mild enzyme deficiency (10% to 60% of normal). Class 4 variants cause very mild or no enzyme deficiency (60% to 100% of normal) [13,16]. How these activity ranges relate to safety of exposure to 8-aminoquinolines is not very clear, nor is the definition of normal, as dis-cussed below.

8-aminoquinolines, malaria, and G6PD deficiency

Primaquine, an 8-aminoquinoline-based drug, is the only available drug recommended by the World Health Organization (WHO) for radical cure of P. vivax infection. The next most advanced product for radical cure is tafeno-quine, which recently completed phase 2 clinical trials.

As a radical cure, primaquine is currently used either in a 7 or 14 day regimen in a doses ranging from 0.25-0.5 mg/kg. For patients with mild to moderate variants of G6PD deficiency, a once-per-week, single 0.75 mg/kg dose of primaquine over eight weeks is recommended, although careful monitoring for hemolysis is also recommended. Unfortunately, none of these regimens is operationally easy to implement. In Brazil and Peru, this has been partially addressed by using a higher-dose, shorter-length primaquine regimen. Tafenoquine as a single-dose radical cure therapy would represent a significant advance in P. vivax therapy. However, a major barrier to widescale adoption of both of these drugs is toxicity in people with G6PD deficiency. While all people exposed to primaquine experience some drop in haemoglobin concentrations [17], people with G6PD deficiency are more likely to experience severe haemolysis, leading to severe haemolytic anaemia and, potentially, death. Despite the availability of primaquine since the 1950s, safety data are scarce.

WHO, confronted with emerging resistance to artemisi-nin and renewed political will to eliminate malaria in many regions of the world, recently released recommendations to administer low doses of primaquine to all patients presenting with falciparum malaria in those settings [18,19]. Based on available data, the new recommended doses are suggested to be low enough to be safe even for G6PD-deficient patients but high enough to have a gametocytocidal effect and block transmission [19,20]. However, before these recommendations can be imple-mented, primaquine will need to be registered in many countries for this use. Uganda and other countries are conducting studies to better understand local prevalence

and types of G6PD deficiency, even within the context of these low doses [2,21].

User requirements and target product profile for G6PD tests

The possible role of G6PD tests within the context of using primaquine for blocking transmission has been discussed elsewhere [2,18,19]. The Bangkok workshop focused on diagnostic tests for G6PD deficiency in P. vivax case management. One breakout session was dedicated to identifying how a patient typically presents with P. vivax infection, how the patient is managed in this scenario, and what type of diagnostic test would be required to support case management. Scenarios were created for Cambodia, India, Myanmar, and Thailand. At least one national malaria control programme representative participated, along with researchers with experience in each country. The different country groups were asked to select a target patient profile, regardless of whether this type of patient carried the highest burden of disease.

In all four settings, it was determined that the target patient would benefit most from a point-of-care G6PD test. There was robust debate over who would use the test and exactly how far into the periphery of the health system the test should go, depending on how complex the treatment algorithm would be. For many cases, based on the fact that many users would have access to a mobile phone and, therefore, some access to electric power, participants felt that some type of automated reader, while not ideal, may be acceptable. While a reader may restrict some access, it can also confer benefits, such as remote monitoring, and it could possibly support some means of recordkeeping [22]. Part of the Bangkok discussion revolved around how often a G6PD test would have to be performed for each individual, and a discussion arose regarding the challenges of record keeping, espe-cially with migrant populations.

Based on this discussion, workshop participants created a generic target product profile (Table 1) [4].

Table 1 Product features of a point-of-care G6PD test in support of radical cure

Features	Ideal	Acceptable	Comments
Test output	Binary, deficient/normal	Quantitative	Presumes a consensus definition of normal that aligns with drug safety
User	Village health workers, mobile malaria workers	District hospital, laboratory worker	This will be defined by national malaria control programmes
Platform	Point-of-care similar to a malaria rapid diagnostic test	A disposable device coupled to a portable, battery-operated device; sensitivity significantly better than human eye	A reader would be acceptable if it significantly improves operational performance
Specimen type	Capillary blood	Capillary blood	Tests must be evaluated for performance with this specimen type
Stability requirements	2 years at 37°C	1 year at 37°C	Expect low throughput at clinic level, so requires small quantities per package or long shelf life
Packaging	Maximum 25 tests per kit	Maximum 25 tests per kit	
Operational temperature range	25-40°C	25-40°C	G6PD enzyme activity is highly temperature dependent (see Figure 2)
Operational humidity range	40-90%	40-90%	None.
Time to result	<10 minutes	<30minutes	Availability of the test result should be aligned with malaria diagnosis and treatment work flow
Read window	>1 hour	10 minutes	Ideally, the test result can be read at any time point after the initial time to result
Sensitivity	Detects all patients (100%) with G6PD activity less than a predetermined cut-off, at or less than which it is unsafe to prescribe a particular dosage of an 8-aminoquinoline	>95% for patients at or less than a defined cut-off G6PD activity	For primaquine, where the fluorescent spot test has been accepted as the standard of care, a 30-40% normal G6PD activity cut-off should be used; for new drugs such as tafenoquine, the cut-off is likely to be higher
Specificity	>95%	>70%	It is preferable to have some patients with normal G6PD activity levels classified as deficient as determined by the Receiver Operating Curve of a diagnostic test
Price	Similar to or less than a malaria rapid diagnostic test	Similar to or less than a malaria rapid diagnostic test	G6PD test represents an additional cost over that of malaria diagnosis and treatment

G6PD product landscape

G6PD activity tests

A survey of products and reagents available for G6PD deficiency testing shows a surprisingly large number of products in the market (more than 20). Available tests determine the G6PD phenotype and overall G6PD activity in a blood specimen, either by direct measurement or through dyes. The outputs can be quantitative, semi-quantitative, or qualitative depending on the platform and assay. Different types of G6PD phenotype assays have recently been reviewed [4,6,23,24].

When workshop attendees were asked which G6PD tests they use, more than 15 products were mentioned, spanning at least three assay platforms. Perhaps the most consolidated G6PD products are those used for newborn screening, which often have high-complexity and sometimes high-throughput platforms [25]. These tests are used in Southeast Asia in national newborn screening due to the high G6PD deficiency prevalence in the region and the risk for infants to develop severe hyperbilirubinaemia, acute bilirubin encephalopathy, and kernicterus [26,27].

Quantitative tests for G6PD activity are considered the gold standard. Yet the predominant standard of care for G6PD deficiency screening is a qualitative test, the fluor-escent spot test, for which there are several commercial kits as well as homebrew assays (assays assembled in the testing laboratory). Beyond those, the wide range of products in the market offer different levels of com-plexity, usability, and performance. Some of these tests have been developed on platforms more suitable for use within the context of malaria case management [28-33]. Overall, with few exceptions [34], there is a paucity of published data that compare G6PD deficiency determin-ation across platforms, and most products on the market have not been evaluated independently.

G6PD genotype tests

G6PD genotype tests characterize the genetic contribution to the G6PD phenotype in a patient. There are several levels at which these tests can be performed, with different degrees of accuracy or resolution. Gel electrophoresis or cytochemical staining can indirectly determine zygosity in females based on whether two G6PD proteins with distinct electrophoretic characteristics or two red cell populations with distinct G6PD activity profiles are observed respectively [35-37]. These are predominantly laboratory-based or homebrew assays. More typically, genotyping is performed through polymerase chain reac-tion (PCR)-based single nucleotide polymorphism (SNP) analysis, and some commercial primer sets are available to determine the genotype through multiplexed PCR. Because not all SNPs can be multiplexed into a single PCR reaction, different panels have been developed based on population prevalence. This genotyping approach is limited to identifying known genotypes and results in severely biased genotype data. Consequently, when both genotyping and phenotyping have been performed on the same patients, the correlation has been mixed [9,38,39]. This is possibly due to different populations experiencing different degrees of polymorphism in this gene and to the severity in G6PD deficiency conferred by the prevalent genotype in a given population.

Sequencing provides the most deterministic G6PD gene characterization, but the G6PD gene—with its 12 introns and 13 exons spanning 18.5 Kb base pairs—is an awkward gene to sequence economically. Given the new sequencing technologies now available, investments should be made in developing multiplexed sequencing assays that look at a range of haemoglobinopathies. Research ethics and consent implications for this type of multi-plexed sequencing assay need to be openly investigated and discussed.

Technical knowledge gaps

To develop G6PD tests that will inform patient manage-ment with 8-aminoquinolines, many questions remain to be answered, both in terms of the G6PD assay itself and the clinical context. Most of these questions revolve around two fundamental issues: (1) defining normal G6PD activity, and (2) defining a G6PD activity cut-off greater than which it is safe to administer a drug at a given.

Defining normal G6PD activity

For the purpose of evaluating diagnostic tests for G6PD deficiency a standard approach for defining an absolute value for normal G6PD activity in a population is required. Ambiguity in how this value is calculated presents practical difficulties in evaluating the performance of G6PD tests, and particularly that of qualitative tests. For qualitative tests, performance will depend on the boundary, or the cut-off point, between normal and deficiency. Typically, G6PD deficiency has been defined as a percentage of normal G6PD activity. In practice, there are almost as many definitions of normal activity as there are publica-tions for evaluating G6PD diagnostic tests [30-33,40,41].

Defining the boundary between normal G6PD activity and G6PD deficiency

Further complicating the issue, there is a paucity of data to correlate definitions for different degrees of G6PD deficiency with risk after exposure to an 8-aminoquinoline challenge [6,42]. This remains a major knowledge gap in understanding G6PD deficiency and the risk of exposure to primaquine and tafenoquine. While it is known that G6PD genotypes differentially impact the response to

primaquine, this knowledge is restricted to only a few of the known G6PD deficiency traits [43,44]. Additionally, acceptable G6PD activity levels for primaquine administration have been defined by the most predominantly used G6PD assay—the fluorescent spot test. This test, by nature of its assay conditions, defines "deficient" at approximately 10% to 30% of normal G6PD activity. As a result, people with severe G6PD deficiency are predominantly excluded from primaquine treatment, whereas most people with mild G6PD activity and most heterozygous women are treated with primaquine. Anecdotally, "this works," but there are no supportive, published data.

If the goal is to expose only patients with normal G6PD activity to 8-aminoquinolines, then the cut-off G6PD activity level would have to be in range of 60% to 70% of normal values, as per the WHO classification. This would also exclude a significant portion of heterozygous women, at least those in whom there are a significant proportion of G6PD-deficient red blood cells.

These two arbitrary definitions or cut-offs have an immense impact on performance requirements for a G6PD test. This is a consequence of the distribution of G6PD activities across a population (Figure 1). Typically, G6PD activity in a population is bimodal, with a minor group of individuals clustered around 10% or less G6PD activity and most clustered in the 60% to 150% range. The 10% to 30% G6PD activity cut-off considered acceptable for primaquine is essentially defined by the fluorescent spot test, a qualitative test for G6PD activity. Thus, developing additional qualitative G6PD tests with similar performance is presumably feasible, though there is a need for improved understanding of the impact of different genotypes on the performance of such qualitative tests against a quantitative test.

By contrast, developing a qualitative G6PD test that accurately excludes patients with less than 60% or 70%

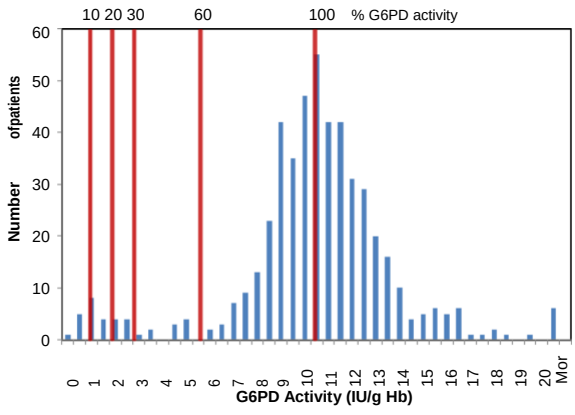

Figure 1 Histogram of G6PD activity for a population described in Table 3; 10%, 20%, 30%, and 60% of the adjusted normal G6PD activity for this population are indicated on the graph.

G6PD activity is likely to be extremely challenging, given the noise-to-signal levels that are likely to exist at this level of activity. A test with discriminatory capabilities in the 60% to 70% cut-off range is likely to require an underlying quantitative or semi-quantitative platform.

Unfortunately, published G6PD test evaluations use inconsistent definitions of normal G6PD activity and also define test sensitivity and specificity based on different cut-off points or degrees of G6PD deficiency. Thus, it is challenging to understand what a qualitative G6PD test defines as normal or deficient and to compare performance claims between publications. Consistent standards for evaluating G6PD tests are sorely needed.

Factors affecting G6PD test performance

Several factors can influence the performance of a G6PD test and its ability to correctly classify a patient as either normal or deficient, starting with the cut-off definition as previously described. These include biological conditions such as concomitant haemoglobinopathies, recent haemo-lytic events that leave a patient with a relatively high proportion of young cells with high G6PD activity that can produce a false normal result, and high leukocyte counts that also lead to a false normal G6PD result. For some of these factors—including a recent malaria infection or other pathological events—it may be possible to predict their effects on a G6PD activity-based assay, but it is still difficult to know how they may affect the risk of an adverse reaction to 8-aminoquinoline exposure. Understanding the impact of haemoglobinopathies and recent haemolytic events on a patient's response to 8-aminoquinolines and the test performance are critical research questions [4].

Because they are enzyme activity tests, the G6PD assays are particularly sensitive to specimen handling and re-action conditions. Specimen integrity is highly sensitive to handling and storage conditions. Acceptable specimen storage conditions for whole blood is up to 14 days at 4°C and for dried blood spots up to 10 days at 4°C or 48–72 hours at room temperature [28,31,45]. Substrate concen-trations and fluctuations in assay temperature influence the enzyme turnover rate. A change of approximately 1 degree in temperature produces a change of 6% in enzyme activity (Figure 2A) [13]. The effect of temperature on G6PD activity values can be accounted for quite effectively by temperature correction factors (Figure 2B). However, in the case of qualitative tests, this may lead to misclassifying deficient specimens as normal if a test is used outside the validated working temperature range (Figure 2C). The combined impact of compromises in specimen collection and operational reaction conditions on the performance of the test in typical malaria treatment settings may result in a wider gap between operational performance of a G6PD test and analytical performance of the test determined under controlled laboratory conditions.

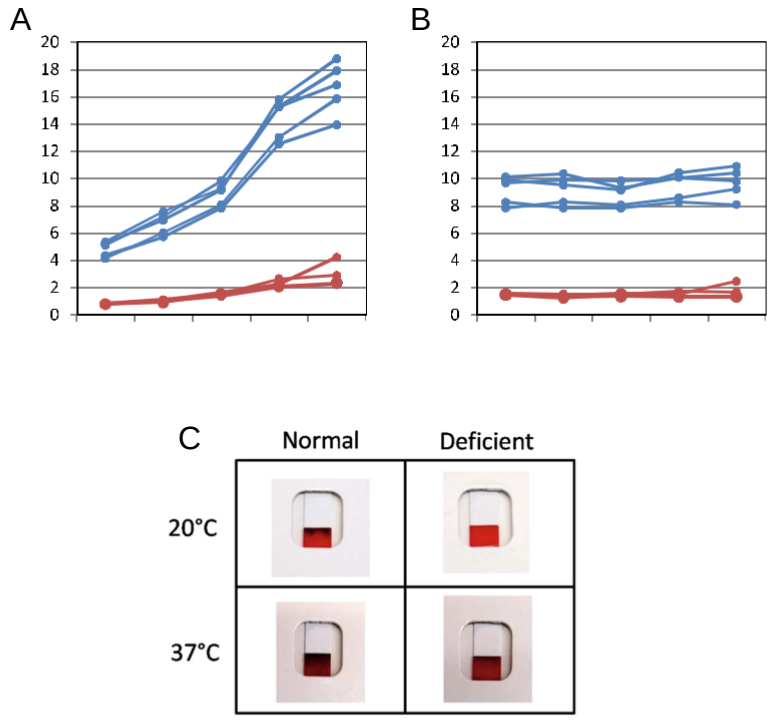

Figure 2 Impact of temperature on G6PD activity-based tests. A. Impact of temperature on quantitative determinations of G6PD activity for five normal and four deficient G6PD samples. B. Normalization of G6PD activity to 30°C through application of the temperature correction factor (Table 2) to values in A. C. Impact of temperature on outputs from a qualitative G6PD test. The deficient sample test result at high temperature looks similar to that of a normal sample at low temperature. Note: the temperature range used for Figure 2C is outside the recommended temperature range in the product insert.

The high proportion of mutations leading to G6PD deficiency affect the stability of the enzyme and specifically the dimer interface [15,46]. Consequently, the dilution factor to which the specimen is subjected in the final assay is also likely to affect the test result and this effect is potentially variant specific (Table 2). Given that the fluorescent spot test is the current standard of care, it will be important to compare the performance of the fluorescent spot test against a quantitative test in different geographical settings to understand this relationship.

In the case of females with heterozygous G6PD alleles, while many display a phenotype of intermediate or mild G6PD deficiency, it is clear from available data that heterozygous women cannot be accurately identified through G6PD enzyme activity assays.

Proposed principles for evaluating diagnostic tests

G6PD tests play a critical safety role in strategies involving radical cure of P. vivax malaria and there is demand for evaluation of the tests. Defining pragmatic guidelines for the evaluation of G6PD tests will be critical to allow comparison of findings between evaluation studies. Below, one approach which would allow meta-analysis of data across sites is suggested. A quantitative test for the gold standard is recommended, but it is also recognized that it is not trivial to implement a G6PD quantitative assay in many field sites.

Study population description

Minimal study population characteristics that need to be assessed for any field evaluation include the proportion of G6PD-deficient cases in the study population, mean

Table 2 Factor by which blood is diluted in the final G6PD activity assay as performed on different G6PD deficiency diagnostic platforms

	Trinity biotech G-6-PDH quantitative test	R&D diagnostics Ltd quantitative test	Trinity biotech G-6-PDH fluorescent spot test	Alere BinaxNOW^{W} Malaria test	Access Bio CareStart™ G6PD deficiency screening test
Initial specimen volume	10 ul	5 μl	10 μl	10 μl	3 μl
Dilution factor	301	80	21	8	41

G-6-PDH: glucose-6-phosphate dehydrogenase.

and median G6PD activity of the study population, and the adjusted male median activity (see below and Table 3). Mean and median values of G6PD activity need to be stratified by gender and adjusted for ambient temperature and the proportion of G6PD-deficient study participants (see below).

If purposive patient recruitment results in inclusion of more G6PD-deficient patients than the local prevalence, mean and median G6PD activity levels also should be provided for the normal males in the study.

Definitions

The definitions provided below are for performance comparison of a qualitative G6PD test to a quantitative G6PD test.

Male median

To minimize the impact of heterozygosity on the definition of G6PD activity, researchers should use the median value of G6PD activity for the entire male population in the study. If purposive or biased recruitment were used for an evaluation, the median G6PD value of the G6PD-normal male recruited for the study should be used as the definition of normal. Otherwise, an adjusted male median calculated as described below should be used.

Adjusted median (100% G6PD activity)

To account for variability in prevalence of G6PD deficiency in a given study population, an adjusted median value is calculated for which males with severe G6PD deficiency (activity less than 10% normal) have been excluded. This is accomplished by:

1. Exclusion of all males with G6PD activity equal to or less than 10% of the male median.
2. Determination of a new median G6PD activity. This is the "adjusted median," which can be used as the 100% G6PD activity value from which cut-off levels are defined.

Table 3 Proposed reference values to describe the G6PD activity profile for a study population

Reference values	Total	Female	Male	Adjusted male
Number of cases	500	282	218	203
Mean (95% CI) U/g Hb	10.23	10.38	10.03	10.72
Standard deviation	2.28	2.10	2.52	1.97
Median (95% CI) U/g Hb	10.33	10.31	10.34	10.70
Range	0-32.25	0.38-32.25	0-24.32	1.50-24.32

CI: confidence interval; Hb: haemoglobin; U: International Unit.
The table is populated with an example data set randomly selected from a true set of quantitative G6PD test results for a population (data kindly provided by Ari Satyagraha).

Cut-off

The percentage of adjusted median at or less than which a patient is classified as positive (G6PD deficient). Samples with G6PD activity greater than the cut-off are considered negative.

True positive (TP)

A sample correctly classified by the diagnostic test under evaluation as having G6PD activity at or less than the cut-off.

False positive (FP)

A sample incorrectly classified by the diagnostic test under evaluation as having G6PD activity at or less than the cut-off.

True negative (TN)

A sample correctly classified by the diagnostic test under evaluation as having G6PD activity greater than the cut-off.

False negative (FN)

A sample incorrectly classified by the diagnostic test under evaluation as having G6PD activity greater than the cut-off.

Range of patients that should be excluded from treatment with 8-aminoquinolines

All patients with G6PD activity less than or equal to the cut-off as determined by the gold standard test (TP + FN).

Range of patients with levels of G6PD activity safe to receive treatment with 8-aminoquinolines

All patients with G6PD activity greater than the cut-off (TN + FP).

Sensitivity

Probability that the test will detect a person with G6PD deficiency.

$$\text{Sensitivity} = \frac{TP}{TP + FN}$$

Specificity

Probability that the test will detect a person with G6PD-normal activity.

$$\text{Specificity} = \frac{TN}{TN + FP}$$

Positive predictive value

Probability that the patient is G6PD deficient when the diagnostic test under evaluation yields a positive result.

$$\text{Positive predictive value} = \frac{TP}{TP + FP}$$

Negative predictive value

Probability that the patient has normal G6PD activity when the diagnostic test yields a negative result.

$$\text{Negative predictive value} = \frac{TN}{TN + FN}$$

Gold standard testing

An established quantitative G6PD test should be implemented as the gold standard test for which 100% G6PD activity and the cut-offs are defined. The quality of the quantitative test should be controlled either through commercially available artificial controls or through samples with known G6PD activity levels. Ideally, this is performed under strict temperature control using venous blood (acid-citrate-dextrose or EDTA anticoagu-lant). If strict temperature control cannot be applied, the temperatures at which the assays were performed should be recorded and then standardized to G6PD activity at 30°C according to temperature correction factors. Some product inserts, such as those for the Trinity Biotech. quantitative test, provide temperature correction factors (Table 4).

Sample size calculations for diagnostic test evaluation

The sample size for evaluations of G6PD tests is driven by the expected performance of the diagnostic test against the predicate gold standard, the local G6PD deficiency prevalence, and the desired accuracy for resulting sensitivity and specificity claims (width of 95% confidence intervals around estimates of sensitivity and specificity). Given the relatively low G6PD deficiency prevalence in most populations worldwide, the sample size is primarily driven by the prevalence and desired accuracy for the evaluation results. Table 5 shows sample calculations for a set of expected test sensitivities over two accuracy constraints and for three G6PD deficiency prevalence rates. In the absence of an appropriate sample size, the statistical power of the study is compromised and the implied uncertainty of the study must be clearly explained.

Table 4 Temperature correction factor as provided in the Trinity quantitative spectrophotometric assay product insert

Cuvette temperature (°C)	Temperature correction factor	Cuvette temperature (°C)	Temperature correction factor
20	1.90	30	1.00
21	1.76	31	0.94
22	1.66	32	0.89
23	1.55	33	0.83
24	1.46	34	0.78
25	1.37	35	0.74
26	1.28	36	0.70
27	1.20	37	0.66
28	1.13	38	0.62
29	1.06	39	0.58

G6PD test performance criteria

In the absence of a more complete understanding of the relationship between risk of haemolysis and level of G6PD deficiency, as well as local G6PD reference values, it is impossible to define a clear normal/deficient G6PD activity cut-off that is consistent and clinically relevant as pertaining to safety and treatment with an 8-aminoquino-line. As a consequence, test performance criteria should be provided for a range of G6PD activity. Percentage of median activity is proposed in order to account for inter-assay and inter-laboratory variability in absolute G6PD activity values. The minimum proposed degrees of deficiency are based on WHO classifications and commonly used ranges: 10%, 20%, 30%, and 60% of the normal male or adjusted median G6PD activity. Absolute cut-off values (in U/g Hb) and sensitivity, specificity, positive predictive value, and negative predictive value should be determined for this range of degrees of G6PD deficiency. Example performance data for the evaluation of a putative G6PD test are described in Tables 3 and 6; the cut-offs are shown in Figure 1.

Regulatory considerations for G6PD testing

The first step toward regulating the quality of G6PD tests will be to define evaluation standards for this class of diagnostic tests. In many countries where G6PD tests are needed to support P. vivax case management, regulatory mechanisms for diagnostic tests are absent, weak, or in transition. In the absence of national guide-lines, some countries default to CE mark and US Food and Drug Administration (FDA) approval. Currently, the BinaxNOW^{W} G6PD test marketed in the United States has obtained FDA approval under 510(k) clearance. Most G6PD tests on the market have at best obtained only CE mark approval.

There is a concern that without clear guidelines for G6PD testing performance criteria, point-of-care G6PD testing will follow a similar route as the malaria rapid diagnostic tests (RDTs), albeit to a smaller scale, wherein a large number of products with varying degrees of quality control and performance entered the market. Variability in RDT quality produced distrust of the product generally,

Table 5 Sample size calculations for evaluation of G6PD diagnostic tests for radical cure

Expected sensitivity	Desired width of CI	Confidence level	Number of disease cases needed	Sample size		
				Prevalence rate 10%	Prevalence rate 15%	Prevalence rate 20%
0.8	0.06	0.95	715	7150	4767	3575
0.8	0.1	0.95	264	2640	1760	1320
0.9	0.06	0.95	417	4170	2780	2085
0.9	0.1	0.95	158	1580	1053	790
0.95	0.06	0.95	238	2380	1587	1190
0.95	0.1	0.95	94	940	627	470
0.96	0.06	0.95	200	2000	1333	1000
0.96	0.1	0.95	81	810	540	405
0.97	0.06	0.95	161	1610	1073	805
0.97	0.1	0.95	68	680	453	340
0.98	0.06	0.95	123	1230	820	615
0.98	0.1	0.95	55	550	367	275
0.99	0.06	0.95	87	870	580	435
0.99	0.1	0.95	44	440	293	220

CI: confidence interval.

and slowed uptake of RDT technology. For G6PD tests, prevention, rather than remediation, of such a problem will likely be less costly for the malaria control and elimination community.

Operational considerations for G6PD testing

Although participants in the workshop's use case scenario session unanimously identified a point-of-care G6PD test as the ideal product profile to support P. vivax case management with 8-aminoquinolines, it does not necessarily follow that:

1. This product profile has a large market demand. The workshop attendees were primarily focused on malaria patients who are the hardest to reach rather than on the largest number of people at risk.
2. This is the best solution for all use cases. As neonatal screening programmes improve in many countries, a more cost-effective approach may be to improve information management systems such that the G6PD status of a patient is more readily available and the need for repeat testing can be minimized.

In Malaysia, neonatal G6PD screening is routinely performed, and G6PD records accompany the patient. In a case where a patient's status is not known, a fluorescent spot test is done, and primaquine is administered based on G6PD status. In contrast, in the Philippines, neonatal screening is supposed to be routinely done but is not universally available, especially to remote and indigenous populations most at risk of malaria infection.

Table 6 Performance results for a putative qualitative diagnostic test modeled against the quantitative results described in Table 3

	10% cut-off	20% cut-off	30% cut-off	60% cut-off
Cutoff value (U/g Hb)	1.07	2.14	3.21	6.42
Number of samples with G6PD levels less than cut-off (percentage)	14 (2.8)	24 (4.8)	28 (5.6)	41 (8.2)
Sensitivity percentage (95% CI)	100	95.8	89.3	68.3
	(73–100)	(77–100)	(71–97)	(52–81)
Specificity percentage (95% CI)	97.1	98.9	99.4	100
	(95–98)	(97–100)	(98–100)	(99–100)
Positive predictive value percentage (95% CI)	0.5	0.82	0.89	1.00
	(0.31-0.69)	(0.62-0.93)	(0.71-0.97)	(0.84-1.00)
Negative predictive value percentage (95% CI)	1.00	1.00	0.99	0.97
	(0.99-1.00)	(0.99-1.00)	(0.98-1.00)	(0.95-0.98)

CI: confidence interval; Hb: haemoglobin; U: International Unit.

The goal of operational research around G6PD deficiency testing and radical cure with 8-aminoquinolines should focus on how to ensure that G6PD status information is available at the point of case management for a patient presenting with P. vivax infection. This may involve linking drug availability to availability of a point-of-care G6PD test, to medical records, or a combination of the two.

Another challenge with introducing and scaling up new G6PD tests is that there are currently few guidelines for adopting and training end users on G6PD testing and counseling. Also, confirming or evaluating operational effectiveness of a G6PD test in clinical settings, as opposed to analytical performance, will be challenging. Additionally an external quality assurance programme will be required. Cost analysis of different approaches to ensuring safe delivery of 8-aminoquinolines should take these factors into consideration, as they may significantly influence cost-effectiveness outcomes.

Market studies segmenting where point-of-care G6PD tests are needed in place of more complex assays will be useful for malaria programmes in terms of resource allocation and for suppliers in terms of understanding the true market size. From the pricing perspective, ideally a G6PD test would be available at the price of a malaria RDT or less. For primaquine, given its low cost, a significantly more expensive test will shift the burden of the cost significantly from treatment costs to diagnostic costs and may impact willingness to pay. Potentially more expensive drugs may tolerate higher prices. From a programme perspective, cost-effectiveness studies should be designed to identify boundaries of these costs.

Conclusions

From a public health perspective, uncertainty remains on whether G6PD testing deficiency status does not need to be taken into account for primaquine-based radical cure in some populations, as reflected in the current WHO guidelines. However, from a patient management perspective, where the individual risk/benefit ratio dictates optimal treatment, knowing the G6PD status of the patient is a prerequisite for prescribing an 8-aminoquino-line-based drug.

Although many questions remain regarding G6PD deficiency and the risk of drug-related adverse events, this should not hinder efforts to evaluate and adopt G6PD tests in support of radical cure. G6PD testing represents an additional cost for malaria treatment and unnecessary G6PD testing should be minimized. Health systems, health management information systems, care-seeking practices, and malaria epidemiology will determine the best way to ensure knowledge of G6PD status for people who have access to 8-aminoquinoline radical cure regimens. While an approach that includes population screening and effective recordkeeping is attractive for the long term, it is clear that point-of-care G6PD testing will be required to meet immediate needs, given that the populations most at risk of P. vivax infection are typically those at the periphery of health care systems and the hardest to reach. In these scenarios, significant operational research will be required to understand how to supply these tests, who the end users should be, how to link the availability of the tests with that of the drugs, and how to implement a recordkeeping system that minimizes the need for repeat testing of individual patients.

A prerequisite to introducing G6PD testing is the availability of high-quality G6PD tests with product profiles that are compatible with end-use cases. Establishing pragmatic and consistent criteria for evaluation of tests should be a high priority. The development and evaluation of new G6PD tests can benefit from the availability of specimen panels [47]. Because factors unique to local populations may affect the performance of G6PD tests, another priority should be to understand the impact of geographical and genetic diversity on the performance of these tests.

Abbreviations

CI: Confidence interval; FDA: US Food and drug administration; FN: False negative; FP: False positive; G6PD/G-6-PDH: Glucose-6-phosphate dehydrogenase; Hb: Haemoglobin; PCR: Polymerase chain reaction; RDT: Rapid diagnostic test; SNP: Single nucleotide polymorphism; TN: True negative; TP: True positive; U: International unit; WHO: World health organization.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

GJD wrote the first draft of the manuscript. All authors contributed to the content, read and approved the final manuscript. LSV is a staff member of the World Health Organization. The author alone is responsible for the views expressed in this publication and they do not necessarily represent the decisions or policies of the World Health Organization.

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Abstract: ABSTRACT
Background
Primaquine is the only currently available drug that clears mature P. falciparum gametocytes thereby preventing malaria transmission to mosquitoes. Concerns about dose-dependent haemolysis in G6PD deficient individuals have limited its use.
Methods
In this randomised double-blinded placebo-controlled trial with four parallel arms, Ugandan children with uncomplicated falciparum malaria and normal G6PD enzyme function were randomised to receive treatment with artemether lumefantrine (AL) plus (1) placebo or (2) 0·1mg/kg, (3) 0·4mg/kg, or (4) 0·75mg/kg (WHO reference dose) primaquine base. The prevalence, density and duration of gametocyte carriage were determined by molecular methods and compared with the reference arm. Haemoglobin concentration and adverse events were determined during 28 days of follow-up.
Findings
A total of 468 participants were randomised to receive AL plus placebo (119), 0·1mg/kg (116), 0·4mg/kg (116) or 0·75mg/kg (117) primaquine base. The mean duration of gametocyte carriage was 6·6 days (95% CI: 5·3-7·8) in the 0·75mg/kg reference arm, similar in the 0·4mg/kg arm (6·3 days; 95% CI:5·1-7·5, non-inferior, p=0·74) but longer in the 0·1mg/kg (8·0 days; 95% CI:6·6-9·4, p=0·14) and placebo arms (12·4 days; 95% CI:9·9-15·0, p<0·001). None of the children showed evidence of treatment-related haemolysis, and the mean maximal fall in haemoglobin concentration was not associated with the dose of primaquine received (p≥0·11).
Interpretation
We conclude that 0·4mg/kg has similar gametocytocidal efficacy to the reference 0·75mg/kg primaquine dose but 0·1mg/kg may have lower efficacy. This calls for the prioritisation of further trials on the efficacy and safety of doses of primaquine between 0·1mg/kg and 0·4mg/kg (including the

recent WHO-recommended dose of 0·25mg/kg), given the potential of widespread deployment to block malaria transmission.

Funding

The trial was funded by the Wellcome Trust (090558), with additional support from the Bill and Melinda Gates Foundation (OPP1034789).

Brief title: Low-dose primaquine for gametocyte clearance

Title: Single-dose primaquine for clearance of *P. falciparum* gametocytes in children with uncomplicated malaria in Uganda: a randomised controlled double-blinded dose-ranging trial

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ABSTRACT

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In this randomised double-blinded placebo-controlled trial with four parallel arms, Ugandan children with uncomplicated falciparum malaria and normal G6PD enzyme function were randomised to receive treatment with artemether lumefantrine (AL) plus (1) placebo or (2) 0·1mg/kg, (3) 0·4mg/kg, or (4) 0·75mg/kg (WHO reference dose) primaquine base. The prevalence, density and duration of gametocyte carriage were determined by molecular methods and compared with the reference arm. Haemoglobin concentration and adverse events were determined during 28 days of follow-up.

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Interpretation

We conclude that 0·4mg/kg has similar gametocytocidal efficacy to the reference 0·75mg/kg primaquine dose but 0·1mg/kg may have lower efficacy. This calls for the prioritisation of further

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Funding

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INTRODUCTION

Effective drug therapy is a key component of malaria control and elimination strategies to reduce both morbidity and onward transmission to mosquitoes.[1] Artemisinin combination therapy (ACT), the current first line treatment in sub-Saharan African countries, achieves excellent cure rates for *Plasmodium falciparum* by rapid clearance of the asexual stages of the parasite. As a consequence of this efficient parasite clearance, ACT reduces the production of malaria transmission stages, gametocytes, and thereby reduces transmission potential.[2] However, onward malaria transmission is not completely prevented because of the limited effect of artemisinins and their partner drugs against mature gametocytes. Mature gametocytes that are present before treatment persist after ACT, often at concentrations below the threshold for detection by conventional microscopy,[3] and may allow onward malaria transmission for up to 14 days after treatment.[3-6]

Primaquine, an 8-aminoquinoline, is the only available drug with well-established activity against mature gametocytes. It clears circulating gametocytes that persist after ACTs, thereby reduces the duration of gametocyte carriage [7-12] and renders most individuals gametocyte-free by day 14 after initiation of ACT-primaquine treatment. [7-9, 12] Primaquine reduces the transmission of malaria to mosquitoes and this effect may precede the clearance of gametocytes [13, 14]. The transmission blocking properties of primaquine were reviewed recently in detail. [15] The World Health Organization has recommended a single dose of primaquine in addition to ACTs for use in two scenarios: for malaria elimination programmes and to stop the spread of emerging artemisinin resistance.[16] Currently, primaquine is recommended for use in first-line antimalarial treatment in many countries.[17]

Despite these recommendations, primaquine is often not deployed because of concerns regarding its haemolytic effect in people with glucose-6-phosphate dehydrogenase (G6PD) deficiency. Primaquine-induced haemolysis may occur after a single dose of primaquine[18] and is dose-

dependent.[19] Because doses of primaquine lower than the WHO-recommended dose may be equally efficacious in clearing *P. falciparum* gametocytes,[15] dose-optimisation for ACT-primaquine is needed.

No formal randomised controlled trials have been conducted to characterise the dose-response relationship of primaquine for *P. falciparum* gametocyte clearance. This study was designed to evaluate the efficacy of reducing doses of primaquine for non-inferiority to the WHO reference 0·75mg primaquine base/kg that has proven efficacy[7, 20] and to assess for superiority of the safety of reducing doses compared to placebo, in individuals with normal G6PD enzyme function.

METHODS

The study was a randomised, double-blinded, placebo-controlled trial with four parallel arms. The study protocol has been described elsewhere in detail.[21] Briefly, the study was conducted at Walukuba Health Centre IV in Jinja district, in eastern Uganda in the period December 2011 to March 2013. In this area, malaria transmission is perennial with seasonal peaks in intensity and an entomological inoculation rate (EIR) of seven infectious bites per person per year was estimated in 2001.[22]

Participants:

Study participants were recruited from children aged 1-10 years attending the Health Centre with fever or history of fever in the last 24 hours, *P. falciparum* mono-infection with a parasite density <500 000/µl, and normal G6PD enzyme function based on a fluorescence spot test (R&D Diagnostics, Aghia Paraskevi, Greece). Exclusion criteria were evidence of severe illness/ danger signs, haemoglobin< 8g/dL, known allergy to study medications, antimalarials taken within the last two days, primaquine taken within the last four weeks and blood transfusion within the last 90 days.

Randomisation masking

Eligible participants were randomly assigned to one of four dose arms: artemether lumefantrine twice daily on days 0 to 2 and, with the fifth dose of AL, a single dose of placebo or primaquine (0·1mg/kg, 0·4mg/kg, or 0·75mg/kg). Four-digit treatment assignment codes were computer-generated by a statistician at LSHTM (EW) and allocated to random dose arms in block sizes of 16. To achieve treatment concealment, a standard volume of masking syrup that concealed the colour and taste of primaquine was added to all doses of primaquine or placebo. Because G6PD deficiency is an X-chromosome linked disorder, randomisation was stratified by gender. Sequential sealed envelopes containing a randomisation code were selected by the study pharmacist from either the male or female pile. The pharmacist was not involved in patient outcome assessment. All other study staff providing care or assessing outcomes, and the participants themselves remained blinded to the intervention arm after assignment.

Procedures:

Interventions: 15mg base primaquine phosphate tablets were crushed and dissolved in 15ml of drinking water to produce a stable 1mg/ml solution and the assigned dose to the nearest 0·5 ml was drawn up by sterile syringe and administered immediately in a plastic cup or spoon. All treatments were administered after a fatty snack (biscuits) and were directly observed. If a participant vomited within 30 minutes, treatment was re-administered. Children who vomited more than three times were excluded from the study and treated for complicated malaria.

Enrolled participants were reviewed on days 0, 1, 2, 3, 7, 10, 14, 21 and 28, or on additional days if they presented at the clinic. There was systematic and prospective assessment for adverse events. New or worsening symptoms, examination findings, or laboratory abnormalities were graded according to a severity scale[23] and causal relationship to the study drug was assessed. A standardised protocol was implemented to detect episodes of haemolytic anaemia.[21] On scheduled visits, approximately 500µL of venous blood was collected for laboratory assessments. On all visits, asexual malaria parasite counts were performed, enumerating parasites per 200 white blood cells; a

hundred microscopy fields were read in the Giemsa-stained thick blood film before a slide was considered parasite negative. At enrolment slides were double-read specifically for gametocytes, following the same procedures as for asexual parasites. Haemoglobin concentration was measured on days 0, 1, 2, 3, 7, 10, 14, 21 and 28 with self-calibrating HemoCue 201+ photometers (HemoCue; Angelholm, Sweden). Gametocytaemia was assessed by quantitative real time nucleic acid sequence based analysis (QT NASBA) using *Pfs25* mRNA[24] on days 0, 2, 3, 7, 10 and, 14. The timing of gametocytaemia measurements was based on previous studies that suggest the gametocyte-clearing effect of primaquine is restricted to the first two weeks after treatment.[7, 25] Nucleic acids were extracted from 50 µL blood samples in L6 buffer [Severn Biotech Limited, Kidderminster, UK] using Total Nucleic Acid Isolation Kits–High Performance [Roche Applied Science, Mannheim, Germany] and a MagNA Pure LC automated extractor [Roche Applied Science, Mannheim, Germany]. The sensitivity of this assay is related to the volume of blood sampled and is in the range of 0·02-0·1 gametocytes/µL for the samples collected [24].

Outcomes and sample size:

The primary outcome measure for efficacy was the non-inferiority of the mean duration of gametocyte carriage in the test doses compared to the reference arm of 0·75mg primaquine base/kg. Secondary outcomes were the point prevalence of gametocytes on days 7, 10, and 14 after treatment, gametocyte circulation time and the area under the curve of gametocyte density over time after primaquine administration. The primary safety outcome was the superiority of the arithmetic mean maximal fall in haemoglobin concentration from enrolment to day 28 of follow up in the primaquine containing arms compared to placebo. Secondary safety outcomes were the superiority assessment of the day of haemoglobin nadir, the maximal percentage fall in haemoglobin, the percentage of participants with haemoglobin less than 5g/dL, requirement for blood transfusion, evidence of black urine, and incidence of severe adverse events.

The sample size calculation took into consideration the primary outcomes for both efficacy and safety. To guide the efficacy calculation we used the QT-NASBA-measured duration of gametocyte carriage in a Tanzanian study which was reduced from 28·6 to 6·3 days (standard deviation 6 days) when primaquine (0·75mg/kg) was added to ACT alone.[25] To assess non-inferiority of the test arms to the reference arm with 80% power, allowing for a 10% loss to follow up, and using a proposed clinically relevant non-inferiority margin of 2·5 days, the target sample size for efficacy was 120 participants per arm. For the safety component, the sample size calculation was based on the mean fall in Hemocue-measured haemoglobin concentration on day 7 after treatment with primaquine 0·6g/dL (standard deviation 1·5) in an earlier Tanzanian study.[18] A sample size of 99 participants per arm would provide 80% power to detect a difference in mean maximal fall in haemoglobin between treatment arms of 0·6g/dL.

Statistical analysis/ methods:

Data were double-entered and transferred into Stata version 12·0 (Statacorp Ltd, Texas, US) for analysis. The duration of gametocyte carriage and gametocyte circulation time were estimated in children with gametocytaemia on day 2, the day of primaquine dosing, using a simple deterministic compartmental mathematical model described by Bousema *et al.* [25] that allows for the release of gametocytes from sequestration and incorporates baseline gametocyte densities in model estimates. The model allows the duration of gametocyte carriage to be estimated as a continuous outcome. As the spacing between sampling times increases some degree of uncertainty is expected, but this was considered to be acceptable for estimates during the first 14 days after initiation of treatment. Treatment arms were compared for non-inferiority to the reference arm using two-sided 95% confidence intervals. As the distribution of gametocyte densities was expected to be skewed, all density analyses involved log-10 transformed data and geometric means were used as summary statistics. The AUC of gametocyte density was assessed per individual using the linear trapezoid method [26] and log-10 transformed. Log AUC was compared to the reference treatment arm using

analysis of variance. Gametocyte point prevalence estimates per treatment arm were compared with the reference arm using the prevalence ratio with 95% confidence intervals. All efficacy analyses were adjusted for gametocyte density at enrolment; the potential effect of gender was tested by adding gender to multivariate models and by stratified analysis.

The primary safety outcome, maximal fall in haemoglobin (g/dL) compared to enrolment value during follow-up, is expressed as an arithmetic mean per treatment arm and pair-wise comparisons made between placebo and each of the primaquine-containing arms, using unpaired *t* tests. The occurrence of adverse events was compared between arms; the significance level was adjusted for multiple comparisons by Bonferroni correction.

Important changes to methods after trial commencement: During the course of review by the trial Data Safety Monitoring Board, the target sample size was reduced to a total 460 participants (i.e. 115 per arm instead of 120) due to a lower than expected loss to follow up.

Role of the funding source This study was funded primarily by a Wellcome Trust Bloomsbury Clinical Fellowship to ACE (090558) and supplemented by funds from the Bill and Melinda Gates Foundation to CD and TB (OPP1034789). The funders had no role in study design, data collection, data analysis, data interpretation or writing the report. The corresponding author had full access to all data in the study. All authors reviewed the manuscript and agreed to submission for publication.

RESULTS

We screened 1215 children with a history of fever and a positive blood smear at Walukuba Health Centre for eligibility to enter the study. The most common reason for exclusion was taking antimalarials in the previous 48 hours. Between December 2011 and December 2012, 468 children were enrolled and randomised, 461 completed treatment and contributed data for the evaluation of safety and efficacy (Figure 1). 36 of these 461 children (7·8%) did not complete 28 day follow-up; the proportion lost to follow-up did not differ significantly between treatment arms, but was higher in the placebo arm. Baseline characteristics are presented in table 1 and were similar in all treatment arms. 43% (199/461) of children were anaemic at baseline (Hb <11g/dL). Treatment failure, assessed clinically and microscopically, was uncommon (table 2) and did not differ between treatment arms (p= 0·68).

Gametocyte prevalence at enrolment was 22·6% (104/461) by microscopy and 81·8% (365/446) by QT-NASBA and did not differ between treatment arms (p=0·91 and p=0·42, respectively). Enrolment gametocyte density was numerically higher in the 0·75mg/kg reference arm but not statistically different from any of the other arms (p≥0·31). Gametocyte prevalence declined after enrolment, 49·3% (170/345) of the individuals who were gametocyte positive at enrolment were still gametocyte positive on day 2 prior to administration of primaquine or placebo. After day 2, the rate of gametocyte clearance was dependent on treatment arm. The mean duration of gametocyte carriage was 6·6 days (95% CI 5·3-7·8) in the reference 0·75mg/kg arm, 6·3 days (95% CI 5·1-7·5) in the 0·4mg/kg arm, 8·0 days (95% CI 6·6-9·4) in the 0·1 mg/kg arm and 12·4 (95% CI 9·9-15·0) in the placebo arm (Table 3). The duration of gametocyte carriage for those gametocyte positive at the moment of primaquine administration was the primary outcome and was tested for non-inferiority to the 0.75mg/kg reference arm. Using the proposed non-inferiority margin of 2·5 days, the 0·4mg/kg arm showed non-inferiority to the reference 0·75mg/kg arm, but the 0·1mg/kg arm did not (being inconclusive for non-inferiority) and placebo was inferior (Figure 2). The mean circulation

time of gametocytes was estimated using Pfs25 QT-NASBA gametocyte density data and indicated a longer circulation time of gametocytes in the 0·1mg/kg arm (p=0·0012) and placebo arm (p<0·0001) compared to the reference 0·75mg/kg arm (Table 3). Gametocyte circulation time was not significantly different between the 0·4mg/kg arm and the reference 0·75mg/kg arm (p=0·80). Compared to the reference arm of 0·75mg/kg, gametocyte prevalence was significantly higher in the 0·1 mg/kg arm on days 7 and 10 and significantly higher in the placebo arm on days 7, 10 and 14 (Table 3). There was no difference in prevalence between the 0·4mg/kg arm and the reference arm throughout follow up (Figure 3). The overall geometric mean gametocyte density was 17·9 gametocytes/µL (95% CI 13·8-23·3) at enrolment, 15·7 gametocytes/µL (95% CI 11·0-22·2)on day 2 before primaquine treatment, 11·6 gametocytes/µL (95% CI 7·2-18·8) on day 3, 5·3 gametocytes/µL (95% CI 3·0-9·3) on day 7, 5·2 gametocytes/µL (95% CI 2·6-10·5) on day 10, and 2·1 gametocytes/µL (95% CI 0·7-5·7) on day 14. This decline in the density of gametocytes in gametocyte positive individuals during follow-up was statistically significant (p<0·001) but densities in gametocyte-positive individuals did not differ significantly between treatment arms on discrete follow up days (data not shown).

The area under the curve of gametocyte density over time, a measure that incorporates both prevalence and density of QT-NASBA estimates, was 3·8 (95% CI 1·7-8·2) gametocytes per µL per day in the placebo arm, 3·8 (95% CI 1·8-7·8) in the 0·1mg/kg arm, 2·1 (95% CI 1·0-4·5) in the 0·4mg/kg arm, and 2·0 (95% CI 0·9-4·3) in the 0·75mg/kg arm (Table 3). After adjustment for gametocyte density at enrolment, the AUC compared to the reference arm was not statistically significantly different for the 0·4mg/kg arm (p=0·79), significantly higher in the 0·1 mg/kg arm (p=0·043), and was non-significantly higher in the placebo arm (p=0·16). None of the efficacy estimates were influenced by the gender of participants.

The mean maximal fall in haemoglobin level did not differ significantly compared to placebo (1·07g/dL; SD 1·11) in the 0·1mg/kg (1·14g/dL; SD 0·94; p=0·61), 0·4mg/kg (1·13g/dL; SD 1·00;

p=0·67), or 0·75mg/kg (1·27g/dL; SD 0·82; p=0·11) arms. The size of the fall in haemoglobin increased with increasing primaquine dose, but this trend was not significant (p=0·46). The timing of the nadir in haemoglobin was independent of treatment arm and the greatest contribution to the total fall in haemoglobin occurred prior to day 2 when the study drug was administered. By day 28, in all treatment arms, haemoglobin had recovered and exceeded baseline levels (figure 4). There were no cases of black water fever, red, black or tea-coloured urine or severe haemolysis and no child required a blood transfusion. There was no impact on safety outcomes by gender.

The proportion of participants experiencing adverse events did not differ between treatment arms after adjustment of significance levels for multiple comparisons. In the gender-stratified analysis, the maximum fall in haemoglobin level appeared larger in the 0.75mg/kg arm compared to placebo arm in females (p=0.023), but this was not statistically significant after Bonferroni correction for multiple comparisons. One child, aged 1.5 years, had a haemoglobin level of less than 5g/dL constituting the single severe adverse event. This male child, who received 0·4mg/kg primaquine, had a baseline haemoglobin concentration of 9·9g/dL. On day 9 of follow up, he had an elective surgical procedure in a mobile clinic. The mother reported no attempt at haemostasis post-operatively and the child had bled severely. By day 14, the haemoglobin had fallen to 4·9g/dL without clinical compromise. Wound care and iron and folate were administered and the haemoglobin recovered to 10·6g/dL on day 28. This event was considered unrelated to the study drug.

DISCUSSION

This is the first formal dose-finding study to assess *P. falciparum* gametocyte clearance following treatment with single-dose primaquine when given in combination with an ACT. We found that the duration of gametocyte carriage was approximately halved when 0·75mg primaquine/kg was given in addition to ACTs. A reduced dose of 0·4mg/kg had a non-inferior gametocytocidal effect compared to the WHO reference dose, while the duration of gametocyte carriage did not reach non-inferiority in the 0·1mg/kg arm and gametocyte prevalence was higher during follow-up. Safety outcomes did not differ significantly between the treatment arms.

In this population with uncomplicated clinical malaria, gametocytes were detected at baseline in 23% of children by microscopy compared to 81% by molecular methods, consistent with previous observations and highlighting the insensitivity of microscopy in identifying potentially infectious individuals.[27] Gametocyte prevalence declined during follow-up; approximately half of the patients with gametocytes at enrolment cleared their gametocytes during the first two days of treatment, before primaquine was given. These dynamics differ from those observed in children a previous ACT-primaquine trial that showed a more gradual decline in gametocyte prevalence after ACT,[7] but resemble those observed recently in symptomatic Kenyan children of the same age-group.[3] Although primaquine shortened the duration of gametocyte carriage, we observed that even the highest single dose of primaquine did not render all individuals gametocyte-negative. In Myanmar and Indonesia, microscopic gametocytes persisted in a small fraction of individuals 21 days after primaquine treatment.[8, 9] In our study, 6% of individuals were gametocyte positive by molecular methods on day 14 after initiation of treatment even with the highest dose of primaquine. The density of these persistent gametocytes was much lower than the density at enrolment. We used gametocyte density estimates for secondary outcome measures because there is no clear lower threshold gametocyte density that is needed for successful mosquito infection.[28-30] The gametocyte circulation time that was calculated based on the rate of decline of gametocyte densities after

treatment, was significantly longer in the placebo and 0·1mg/kg arm but not significantly different in the 0·4mg/kg arm compared to the reference 0·75mg/kg arm. The area under the curve of gametocyte density over time, a summary measure for malaria transmission potential, [7, 26, 31] was not significantly different between the 0·4 and the 0·75mg/kg dose group but was numerically higher in the 0·1mg/kg dose and placebo arm, compared to the reference dose of 0·75mg/kg. Baseline differences between treatment arms in asexual parasite density did not result in differences in baseline gametocyte prevalence or density or differences in treatment outcome and did not confound the comparison of gametocyte dynamics between treatment arms.

While our trial used sensitive molecular gametocyte detection tools and thereby provides a level of detail that is lacking in most other primaquine trials, a relevant shortcoming of this study and other studies is that gametocyte infectiousness to mosquitoes was not determined. A proportion of the gametocytes that are observed by microscopy shortly after primaquine treatment may be non-infectious.[15] It is currently unknown whether Pfs25 mRNA can be detected from non-viable gametocytes and it is plausible that a proportion of the gametocytes that we detected were non-infectious. We may, therefore, have underestimated the transmission-blocking effect of primaquine. None of the currently available gametocyte detection tools allow inferences on the infectiousness of gametocytes to mosquitoes to be made and only mosquito feeding assays can provide definitive evidence for the transmissibility of gametocytes. There are, however, limitations in the extent to which labour-intensive mosquito feeding assays can be used in clinical trials.[32] While gametocyte measurements can be conducted repeatedly from the same individual, the handful of clinical trials that have used mosquito feeding assays typically perform feeding experiments on a single time-point per individual [3, 33, 34] and thereby ignore the dynamics of gametocyte infectivity [34]. Future studies that confirm the gametocytocidal effects of low dose primaquine should therefore preferentially include mosquito feeding assays at intervals during follow up.

A further limitation of this study was the lack of available paediatric dose formulations for primaquine, which necessitated titration of crushed primaquine in solution for accurate dosing. Whilst the approach of using crushed tablets has been used previously for the 0·75mg/kg dose, [7,8] *it* is conceivable that this may have impacted efficacy particularly of the 0.1mg/kg dose. Further data on the relative bioavailability of different formulations of primaquine are required. Hence, a pre-requisite to the up-scaling of primaquine deployment will be the availability of reliable paediatric formulations for low dose primaquine. This study aimed to determine the safety of low-dose primaquine in individuals with normal G6PD enzyme function. G6PD deficient individuals were excluded in this study based on the fluorescent spot test, the most widely used enzyme function test[13] that detects enzyme function to a cut off of approximately 20-30% of normal activity.[35]. We chose to exclude G6PD deficient individuals to first establish the lowest efficacious dose before vulnerable individuals are exposed to a potentially haemolytic drug. Although haemolysis has been observed in individuals without common mutations in the G6PD enzyme,[36] the exclusion of individuals with abnormal enzyme function obviously limits the generalisability of the safety outcomes of this study and this needs to be addressed in future studies. Given this caveat, haemoglobin fell most rapidly in the first two days after enrolment in all study arms, implying that the greatest effect on haemoglobin was due to clinical malaria rather than a drug effect. Thereafter, haemoglobin recovered to pre-morbid levels. A similar trend was found in Tanzanian children,[7] in Myanmar,[37] and in Indonesia.[9] We found no children with objective measures of clinically significant haemolysis, black urine, or requirement for hospital admission or blood transfusion. The single severe adverse event was in a child who underwent an elective surgical procedure unrelated to the clinical malaria episode on day 9 and therefore after the expected duration of primaquine-associated haemolysis.

In this dose-finding trial, primaquine administration was delayed until day 2 after initiation of schizonticidal therapy. This is when, in the context of uncomplicated malaria, the rate of malaria-attributable haemolysis is expected to be declining, and comparisons of haematological effects

between dose arms are expected to be less affected by the effects of acute malaria infection. In operational terms, administering primaquine on the first day of schizonticidal treatment is likely to be advantageous and comparisons of the efficacy of day 0 versus day 2 administration will be important.

The World Health Organization has recommended the use of a single dose of 0·75mg primaquine base/kg in combination with schizonticidal drugs to reduce transmission of malaria for over 40 years.[38] However, no dose-finding trials underpinned this recommendation. The limited evidence base for primaquine use has prompted uncertainty as to the benefit of an intervention which carries a documented risk of haemolysis in malaria endemic populations.[39, 40] The real threat of spreading artemisinin resistance[41] has led to urgency in addressing this problem. In September 2012, whilst the current study was ongoing, an Evidence Review Group commissioned by the World Health Organization revised its recommended dose to 0·25mg primaquine base/kg to be added to ACT to treat parasitologically-confirmed falciparum malaria infection in new programmes for malaria elimination and to stop the spread of artemisinin resistance.[42] This dose revision was based on under-powered historical studies and the need for contemporary data was highlighted [43]. The 0·25mg/kg dose was not evaluated in the current study. This is a limitation of the current study and leaves important questions for future dose-findings studies. However, we have shown that gametocytocidal efficacy is retained when the primaquine dose is reduced from 0·75mg/kg to 0·4mg/kg and that a dose-response relationship exists for lower doses. The finding of a reduced gametocytocidal efficacy below 0.4mg/kg appears to contradict suggestions of uniform efficacy in the range of 0.065 and 0.75mg PQ/kg (ref 16). This novel information provides a valuable starting point in identifying the most efficacious and safe low dose of primaquine for transmission blocking. The subsequent evaluations of primaquine should include assessments of i) the efficacy of doses less than 0·4mg/kg (including the newly-recommended 0·25mg/kg dose), using mosquito transmission endpoints to allow for differences in infectiousness of gametocytes persisting after treatment, ii) the optimal timing of primaquine in combination with ACT, iii) the pharmacokinetics of low-dose

primaquine and iv) the safety of low dose primaquine in individuals with G6PD enzyme deficiency, which is of high priority. Because of differences in gametocyte dynamics between African and Asian settings [44] and differences in the severity of G6PD deficiency between geographical regions,[45] studies in a range of malaria endemic settings are needed.

Panel: Research in context

Systematic review

We searched PubMed in May 2013, without date or language restrictions, with the terms "primaquine" AND "malaria, falciparum" AND "gametocyte" OR "primaquine" AND "malaria, falciparum" AND "transmission". We found no randomised controlled trials evaluating the dose-response relationship of primaquine for gametocytocidal activity. A Cochrane review of the transmission-reducing efficacy of primaquine published in September 2012 found five trials evaluating a primaquine-ACT combination that satisfied the criteria for inclusion and none of these evaluated a range of doses.[40] Three studies have assessed the haematological safety of primaquine with ACTs,[7, 36, 37] but this trial is unique in having been specifically powered to assess safety outcomes.

A search of clinical trial registration sites for primaquine dose-finding trials for transmission blocking revealed one single trial that is underway in the Gambia (NCT01838902) to assess the efficacy of ACT alone, 0.2mg/kg, 0.4mg/kg and 0.75mg/kg primaquine base in asymptomatic individuals and this trial is scheduled for completion in 2015. Another study (NCT01743820) is in development to assess primaquine dose escalation from 0.125mg/kg in a total of 50 participants randomized over different dosing arms. Several other registered studies with primaquine for *P. falciparum* do not involve dose-finding but will address relevant questions for future wide-scale deployment of primaquine. These studies include a study on the optimal timing of primaquine administration (NCT01906788, trial recruiting), primaquine pharmacokinetics (NCT01552330, NCT01525511 both completed august

2013) and mosquito feeding as an endpoint comparing ACT alone with 0.75mg/kg primaquine (NCT01849640 not yet recruiting, with a scheduled three-year timeline).

Interpretation

This is, to our knowledge, the first randomised, placebo-controlled trial to evaluate the dose-response relationship of single-dose primaquine for gametocyte clearance and for safety in falciparum malaria. This trial was conducted in African children with clinical malaria and normal G6PD enzyme function. A dose reduction to 0·4mg/kg primaquine base had demonstrable non-inferiorityto the reference 0.75mg/kg dose, whilst a dose of0.1mg/kg did not achieve non-inferiority. This trial was designed and initiated prior to a revision of the WHO guidelines recommending 0.25mg/kg primaquine for transmission blocking, in the light of which, this new dose must now be evaluated. In this population, all doses of primaquine had similar safety profiles to placebo. An assessment of low dose primaquine in G6PD deficient individuals is warranted.

CONTRIBUTORS

ACE, CD, TB, NJW, SGS, SY and ELW designed the study and were involved in interpretation and writing. ACE implemented and led the study. ELW and JB provided statistical support for data analysis. AO and GG were involved in data collection. LG and KHWL performed the QT-NASBA laboratory analysis. MK, HW, AM and SN provided logistical support. All authors reviewed and approved the final manuscript.

CONFLICTS OF INTEREST

All authors declare that we have no conflicts of interest.

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Ethical approval

Ethical approval was granted for the trial protocol and informed consent forms by the Makerere University School of Medicine Research Ethics Committee (protocol 2011-210), the Uganda National Council of Science and Technology (protocol HS1056) and the London School of Hygiene and Tropical Medicine research ethics committee (protocol 5987). The importation of the study drug was approved by the Ugandan National Drug Authority. The trial Data Safety Monitoring Board (DSMB) and Trial Advisory Committee was convened prior to the start of the trial and met at predetermined stages of the study. Consultations with local community stakeholders in Walukuba were held before, during and after trial completion.

TABLES AND FIGURES

Table 1: Table 1: Baseline characteristics of study participants.

Legend for table 1:

AL = artemether lumefantrine; PQ = primaquine; IQR = Interquartile range (25^{th}-75^{th} percentile); SD = standard deviation; GM = geometric mean

Table 2: Treatment outcomes for the different regimens on day 28 after initiation of treatment.

Legend for table 2:

*p value for comparison with placebo, using chi-squared or Fisher's exact tests Outcomes are unadjusted by PCR.

AL = artemether lumefantrine; PQ = primaquine; ITT = intention to treat; ACPR = adequate clinical and parasitological response; ETF = early treatment failure; LTF = late treatment failure. Definitions of ACPR, ETF and LTF are according to WHO Methods for Surveillance of Antimalarial Drug Efficacy 2009.

Table 3: Efficacy outcome: gametocyte carriage during follow-up for the different treatment regimens

Except for the duration of gametocyte carriage, all estimates were adjusted for gametocyte density at enrolment.

*p value for comparison with reference 0·75mg/kg treatment arm.

†calculated for all children who had gametocytes on the day of primaquine/ placebo administration.

AL = artemether lumefantrine; PQ = primaquine;

Figure 1: Trial profile

Legend for figure 1:

AL=artemether lumefantrine; PQ=primaquine; ITT=intention to treat. AL was administered as six doses over 3 days (days 0, 1, 2); PQ or placebo was given together with the fifth dose of AL on the morning of day 2.

The two post-treatment exclusions (due to delayed confirmation of parasitaemia) were followed up for safety

Figure 2: Mean duration of gametocyte carriage by treatment regimen

Legend for figure 2:

The duration of gametocyte carriage was estimated by fitting a simple deterministic compartmental mathematical model to repeated Pfs25 QT-NASBA gametocyte prevalence estimates. Artemether-lumefantrine (AL) was administered as six doses over 3 days (days 0, 1, 2); PQ or placebo was given together with the fifth dose of AL on the morning of day 2. Symbols indicate the mean duration of gametocyte carriage, error bars the upper and lower limit of the 95% confidence interval. The dashed line indicates the set threshold for non-inferiority compared to the 0·75mg/kg reference arm of 2·5 days.

Figure 3: Gametocyte prevalence (a) and prevalence ratio (b) for each treatment regimen during the14 day follow up period

Legend for figure 3:

Gametocyte prevalence during follow-up. The prevalence of gametocytes determined by Pfs25 QT-NASBA is given in the upper panel. Error bars indicate the upper limit of the 95% confidence interval;

asterisks indicate a statically significant difference compared to the reference 0·75mg/kg arm. The lower panel shows the odds ratio of gametocyte prevalence on each of the days of follow-up compared to the reference 0·75mg/kg arm after adjustment for baseline gametocyte density. Error bars indicate the upper and lower limits of the 95% confidence interval. Artemether-lumefantrine (AL) was administered as six doses over 3 days (days 0, 1, 2); PQ or placebo was given together with the fifth dose of AL on the morning of day 2.

Figure 4: Mean change in haemoglobin measurements by treatment regimen during follow up

Legend for figure 4:

Haemoglobin concentrations (g/dL) during follow up are expressed relative to that at enrolment for Artemether-lumefantrine (AL)-placebo (black symbols and dashed line), AL-primaquine 0·1mg/kg (pink symbols and solid line), AL-primaquine 0·4mg/kg (orange symbols and dashed line) and AL-primaquine 0·75mg/kg treatment regimens (red symbols and solid line). AL was administered as six doses over 3 days (days 0, 1, 2); PQ or placebo was given together with the fifth dose of AL on the morning of day 2.

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Table

Table 1: Baseline characteristics of study participants

	AL + Placebo	AL + PQ 0·1mg/kg	AL + PQ 0·4mg/kg	AL + PQ 0·75mg/kg
N	117	115	113	116
Gender, % male (n/N)	48·7 (57/117)	49·6 (57/115)	49·6 (56/113)	49·1 (57/116)
Age, median (IQR)	5·0 (3·0-7·5)	5·0 (3·3-7·0)	5·3 (3·2-7·0)	4·1 (3·0-7·0)
Bodyweight, median (IQR)	16·0 (13·0-20·5)	16·0 (13-22)	17·0 (14-23)	15·0 (13-19)
Body temperature, mean °C (SD)	38·0 (1·0)	38·3 (1·1)	38·0 (1·2)	38·2 (1·1)
Haemoglobin, mean g/dL (SD)	11·3 (1·5)	10·9 (1·5)	11·2 (1·5)	11·2 (1·4)

Asexual parasite density, GM parasites/μL(IQR)	17661 (5260-65130)	18420 (4440-92780)	16457 (3260-81240)	32497 (10880-151180)
Gametocyte prevalence by microscopy, % (n/N)	23·1 (27/117)	24·4 (28/115)	20·4 (23/113)	22·4 (26/116)
Gametocyte prevalence by QT-NASBA, % (n/N)	78·5 (91/116)	86·0 (98/114)	77·5 (86/111)	81·3 (91/112)
Gametocyte density by QT-NASBA, GM (IQR)	38·4 (5·6-302·8)	37·8 (12·6-149·1)	38·0 (1·0-190·4)	79·8 (25·4-245·7)

Table 2: Treatment outcomes for the different regimens on day 28 after initiation of treatment.

Outcome	Details	AL + Placebo	AL + PQ 0·1mg/kg	p value*	AL + PQ 0·4mg/kg	p value*	AL + PQ 0·75mg/kg	p value*
Number evaluated		117	115		115		116	
Day 28 treatment outcomes								
Excluded from ITT analysis, % (n)	Withdrawal unrelated to study drug or malaria	0	0		2		0	
	Lost to follow up	15	7		7		5	
ACPR, % (n)	Day 28:	96·1% (98)	93·5% (101)	0·41	100% (106)	0·12	95·5% (106)	0·83
Treatment failures, % (n)	ETF	0	0		0		0	
	LTF (day 28)	3·9% (4)	6·5% (7)	0·41	0	0·12	4·5% (5)	0·83

Table 3: Efficacy outcome: gametocyte carriage during follow-up for the different treatment regimens

Treatment	**AL + Placebo**	**p value***	**AL + PQ 0·1mg/kg**	**p value***	**AL + PQ 0·4mg/kg**	**p value***	**AL + PQ 0·75mg/kg**
Mean duration of gametocyte carriage in days (95% CI) †	12·4 (9·9-15·0)	<0·0001	8·0 (6·6-9·4)	0·14	6·3 (5·1-7·5)	0·74	6·6 (5·3-7·8)
Mean circulation time in days, per gametocyte (95% CI)	1·97 (1·64-2·31)	<0·0001	1·47 (1·22-1·73)	0·001	0·95 (0·77-1·13)	0·80	0·98 (0·78-1·18)
Gametocyte prevalence on day 7, % (n/N)	34·8 (40/115)	0·001	23·1 (25/108)	0·044	10·6 (11/104)	0·47	14·4 (15/104)
Gametocyte prevalence on day 10, % (n/N)	20·5 (23/112)	0·008	16·8 (18/107)	0·020	9·3 (10/107)	0·46	7·4 (8/108)
Gametocyte prevalence on day 14, % (n/N)	15·2 (16/105)	0·017	5·8 (6/103)	0·72	2·9 (3/103)	0·51	5·7 (6/107)

Figure 1 revised trial profile

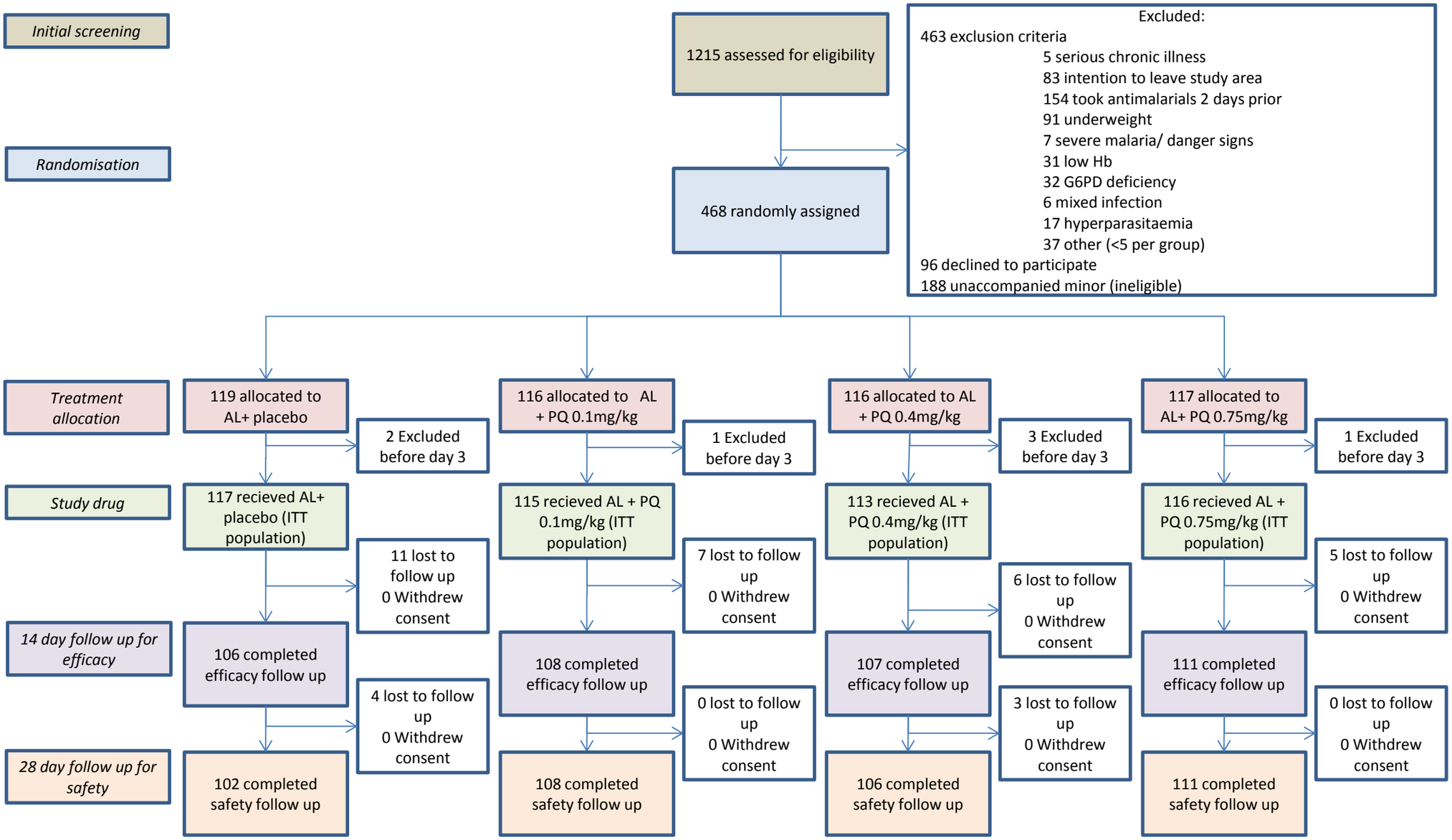

Figure 2 gametocyte durations
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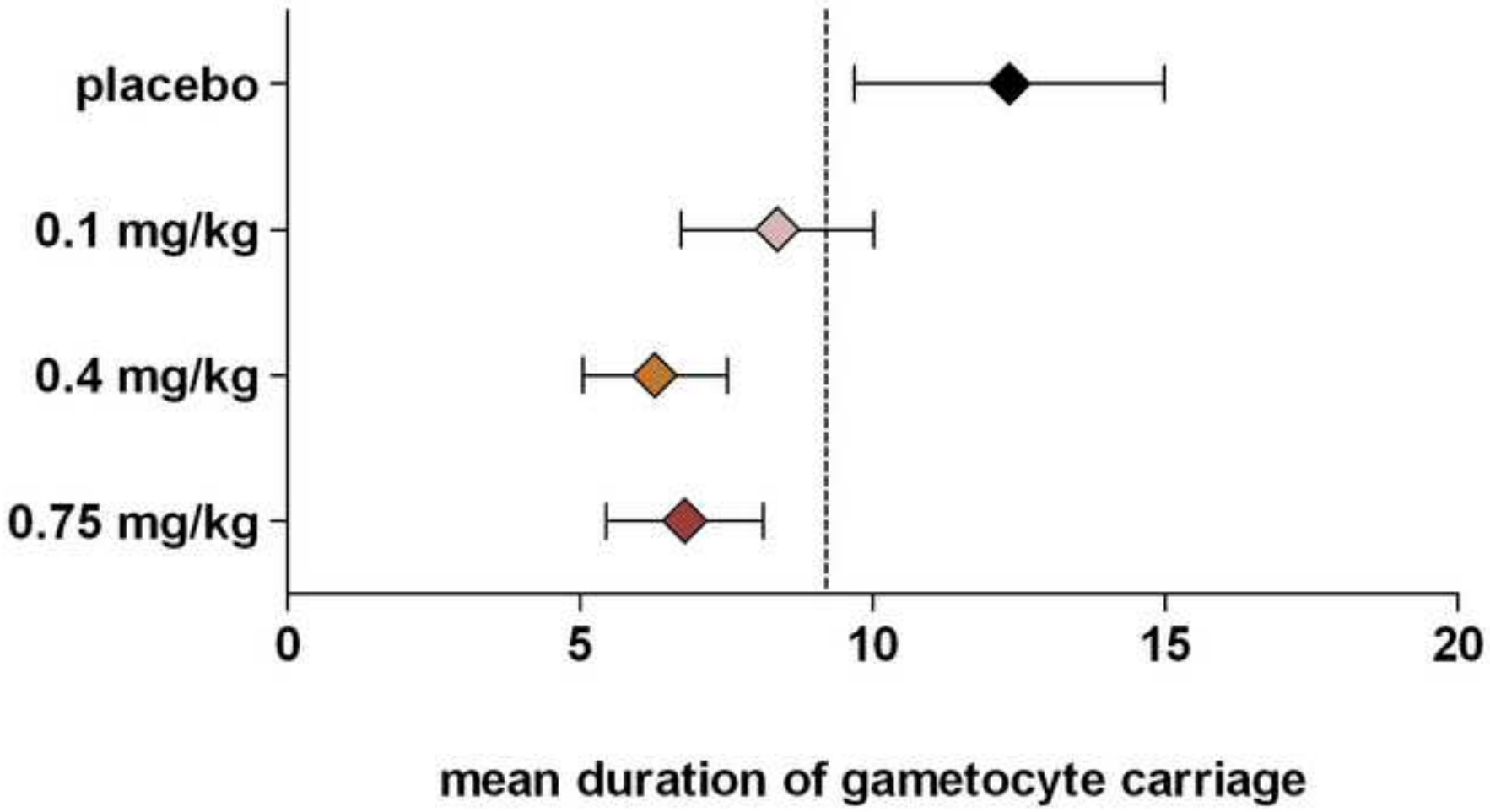

Figure 3 revised gametocyte prevalence tiff
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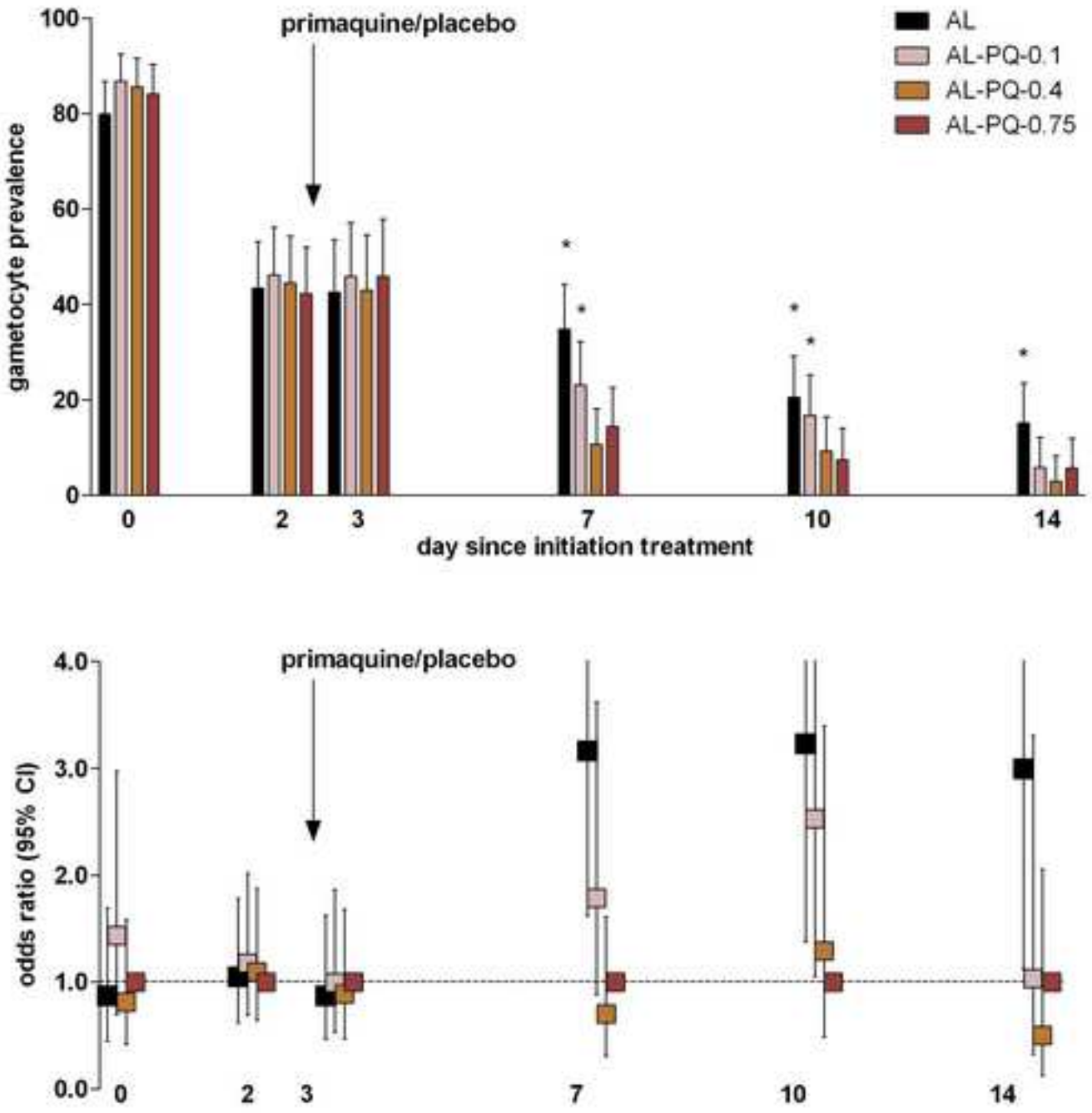

Figure 4 revised safety

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